METHODS FOR SECRETORY PRODUCTION OF PROTEINS

Background of the Invention

[0001] The present invention relates to a method of producing a heterologous protein efficiently by secretory production.

[0002] A number of methods for the secretory production of heterologous proteins have been previously reported such as those described in the review on the secretory production of a heterologous protein by a bacterium belonging to the genus Bacillus [Microbial. Rev., 57, 109-137 (1993)], the review on the secretory production of a heterologous protein by methylotrophic yeast *Pichia pastoris* [Biotechnol., 11, 905-910 (1993)] and the report on the industrial production of heterologous proteins by the mould belonging to the genus Aspergillus [Biotechnol., 6, 1419-1422 (1988); Biotechnol., 9, 976-981 (1991)].

[0003] The transglutaminase produced by the secretory production according to one embodiment of the present invention is an enzyme which catalyzes acyltransfer reaction of γ-carboxylamide groups in the peptide chain of the protein. When the enzyme is reacted with a protein, the formation of the cross-linkage ε-(γ-Glu)-Lys and the replacement of Gln with Glu by deamidation can occur. Transglutaminase has been used to manufacture gelled food products such as jelly, yogurt, cheese or gelled cosmetics and others, and to improve the quality of meat and the like (Japanese publication of examined application No.1-50382). Moreover, transglutaminase is an enzyme having industrially high usefulness in that it has been used to manufacture materials for thermostable microcapsules, carriers for immobilized enzymes, etc..

[0004] Transglutaminases derived from animals and from microorganisms (microbial transglutaminase: referred to as 'MTG' hereinafter) have been previously known. The former is a calcium ion-dependent enzyme that is distributed in animal organs, skin, blood, etc.. The examples include guinea pig hepatic transglutaminase (K. Ikura et al. Biochemistry 27, 2898 (1988)), human epidermal keratinocyte transglutaminase (M. A. Phillips et al. Proc. Natl. Acad. Sci. USA 87, 9333 (1990)),

human blood coagulation factor XIII (A. Ichinose et al. Biochemistry 25, 6900 (1990)) and others.

[0005] For the latter, calcium-independent transglutaminases have been discovered from bacteria belonging to the Streptoverticillium genus, which include, for example, *Streptoverticillium griseocarneum* IFO 12776, *Streptoverticillium cinnamoneum* sub sp. cinnamoneum (hereinafter, *S. cinnamoneum*) IFO 12852, *Streptoverticillium mobaraense* (hereinafter, *S. mobaraense*) IFO 13819 and others (Publication of unexamined Japanese patent application JP-Kokai No. 64-27471). The peptide mapping and the structural analysis of the genes revealed that the primary structure of the transglutaminase produced by these microorganisms shared no homology with transglutaminases from animals (European Patent Application Publication No. 0 481 504 A1).

[0006] Because microorganism-derived transglutaminases (MTG) are produced through the purification from the cultures of microorganisms such as described above, there have been problems in terms of the amount and the efficiency and the like. The production of transglutaminase using a genetically engineered procedure has also been attempted. Transglutaminase proteins and the genes thereof have been reported in, for example, Biosci. Biotechnol. Biochem., 58, 82-87(1994); Biosci. Biotechnol. Biochem., 58, 88-92(1994); Biochimie, 80, 313-319(1998); Eur. J. Biochem., 257, 570-576(1998); WO 96/06931; WO 96/22366, etc., which report the expression and production of transglutaminase in host-vector systems such as *Streptomyces lividans*, *Aspergillus oryza*e and *Escherichia coli*. In addition to this information, a method has been reported wherein a transglutaminase is produced by secretory production in microorganisms such as *E. coli* and yeast (JP-Kokai No. 5-199883) and a method has been reported wherein active MTG is produced by expressing MTG as an inactive fused protein in an inclusion body within *E. coli* and subsequently solubilizing the inclusion body using protein-denaturing agents, and then, reconstituting it through the removal of the denaturing agents (JP-Kokai No. 6-30771). However, the problem has been noted that the expression level is significantly low in the secretory

production by microorganisms such as *E. coli* or yeast.

[0007] On the other hand, there are examples of previous studies for the efficient secretory production of heterologous proteins using a coryneform bacterium including the secretion of nucleases and lipases [US4965197; J. Bacteriol., 174, 1854-1861(1992)] and the secretion of proteases such as subtilisin [Appl. Environ. Microbiol., 61, 1610-1613 (1995)] by Corynebacterium glutamicum (hereinafter, C. glutamicum), a study on the secretion of cell surface proteins of a coryneform bacterium [International patent application published in Japan No. 6-502548], the secretion of fibronectin-binding protein using this study [Appl. Environ. Microbiol., 63, 4392-4400 (1997)], a report wherein the secretion of proteins was enhanced using a mutated secretory machinery [JP-Kokai No. 11-169182], etc., but there has been a limited number of reports on limited proteins. In light of the accumulated amount of proteins, Appl. Environ. Microbiol., 61, 1610-1613 (1995) describes that about 2.5 mg/ml of protein was accumulated by expressing the alkaline protease gene from *Dichelobacter nodosus* in *C. glutamicum* using a promoter of subtilisin gene(aprE) from Bacillus subtilis, ribosome binding site and the sequence of a signal peptide, but US4965197; JP-Kokai No. 6-502548; and JP-Kokai No. 11-169182 do not specifically describe the values of the amount of the proteins secreted and accumulated. Furthermore, in the case of the fibronectin-binding protein [Appl. Environ. Microbiol., 63, 4392-4400 (1997)], only the secretory accumulation of the protein of about 2.5μg/L is confirmed. Thus, there have been no reports that heterologous proteins could be efficiently accumulated in the medium at a practical level.

[0008] Additionally a genetic engineering technology for a coryneform bacterium has been developed in the system using plasmid and phage, such as the establishment of the transformation by protoplast [J. Bacteriol., 159, 306-311(1984); J. Bacteriol., 161, 463-467(1985)], the development of a various type of vectors [Agric. Biol. Chem., 48, 2901-2903(1984); J. Bacteriol., 159, 306-311(1984); J. Gen. Microbiol., 130, 2237-2246(1984); Gene, 47, 301-306(1986); Appl. Microbiol. Biotechnol., 31,

65-69(1989)], the development of the regulation method of gene expression [Bio/Technology, 6, 428-430(1988)] and the development of cosmid [Gene, 39, 281-286(1985)]. Moreover there are reports on the cloning of genes from a coryneform bacterium [Nucleic Acids Res., 14, 10113-1011(1986); J. Bacteriol., 167, 695-702(1986); Nucleic Acids Res., 15, 10598(1987); Nucleic Acids Res., 15, 3922(1987); Nucleic Acids Res., 16, 9859(1988); Agric. Biol. Chem., 52, 525-531(1988); Mol. Microbiol., 2, 63-72(1988); Mol. Gen. Genet., 218, 330-339(1989); Gene, 77, 237-251(1989)].

[0009] Furthermore, a transposable element derived from a coryneform bacterium has also been reported [WO93/18151; EP0445385; JP-Kokai No. 6-46867; Mol. Microbiol., 11, 739-746(1994); Mol. Microbiol., 14, 571-581(1994); Mol. Gen. Genet., 245, 397-405(1994); FEMS Microbiol. Lett., 126, 1-6(1995); JP-Kokai No. 7-107976].

[0010] The transposable element is a DNA fragment that can be transposed on the chromosome and is known to be present in a wide range of organisms ranging from prokaryotes to eukaryotes. Transposons using transposable elements have been developed [WO93/18151; JP-Kokai No. 7-107976; Mol. Gen. Genet., 245, 397-405(1994); JP-Kokai No. 9-70291] and a heterologous gene is able to be expressed using a transposon.

Summary of the Invention

[0011] The object of the invention is to provide a method for the production of a heterologous protein by making a coryneform bacterium to produce an industrially useful heterologous protein, for example, transglutaminase, and efficiently secrete the product extracellularly (i.e., secretory production).

[0012] The inventors of the present invention found a mutant which had remarkably higher production capacity in the production of heterologous proteins using coryneform bacteria compared to the wild type *Corynebacterium glutamicum* ATCC13869, which led to the present invention.

[0013] Accordingly, the present invention is a method of producing heterologous proteins characterized in that a fusion protein is produced and secreted (secreto-produced) by a mutant coryneform bacterium which has a capacity of secreting the heterologous protein, which is connected to the downstream of the signal peptide from a coryneform bacterium, at least 2-fold higher than the wild type *Corynebacterium glutamicum* ATCC13869.

[0014] More specifically, the invention is a method to obtain a large amount of an intended heterologous protein, for example, transglutaminase, by introducing a genetic expression construct into a coryneform bacterium, culturing the thus transformed coryneform bacterium, efficiently secreting the resulting protein extracellularly and recovering the released protein, wherein the genetic expression construct contains a gene sequence encoding an intended protein which is ligated to the downstream of a sequence encoding the signal peptide derived from a coryneform bacterium, especially the signal peptide of a cell surface protein.

[0015] As used herein, "the secretion" of a protein or peptide refers to the transportation of the protein or peptide molecule outside the bacterium cell (extracellular transportation) including the case where the protein or peptide molecule exist finally in completely free form in the medium as well as the case where only the part of the protein or peptide molecule is present outside the cell and the case where they are located on the surface of the cell.

Description of the Preferred Embodiments

[0016] According to the method of the invention, a coryneform bacterium is used as a host vector system, and a large amount of an extracellularly secreted interested protein may be obtained by generating an expression construct wherein the gene encoding the interested protein is ligated to the downstream of the signal peptide of cell surface protein from coryneform bacterium, introducing the construct into a coryneform bacterium and expressing it.

[0017] The proteins which may be secreto-produced by the method of the present

invention include enzymes, physiologically active proteins and peptides which are industrially useful. Transglutaminase, which is secreto-produced in one embodiment of the present invention, is widely used in the food processing, the manufacture of pharmaceuticals and the like.

[0018] A secretory protein has been generally known to be translated as a prepeptide or prepropeptide and thereafter to be formed into a mature protein. That is to say, in general, it has been known that it is translated as a prepeptide or prepropeptide, then the signal peptide ("a pre-part") is cleaved, thereby it is converted into a mature peptide or propeptide by further cleaving of the pro-part with a protease. As used herein, "a signal sequence" refers to the sequence which is located at the N-terminal of a secretory protein precursor and which is not present in a naturally occurring mature protein, and "a signal peptide" refers to the peptide which is cleaved from such a protein precursor. Generally, a signal sequence is cleaved coupling the extracellular secretion by a protease (generally referred to signalpeptidase). Although such a signal peptide shares certain common features in the sequence over species, a signal peptide which has secretory function in one species does not necessarily have the same secretory function in another species.

[0019] As used herein, a protein which contains both a signal peptide and a pro-part, that is, a primary translational product can be referred to "a preproprotein," and a protein which does not contain a signal peptide but does contain a pro-part can be referred to "a proprotein." A pro-part of a proprotein can be referred to as "a pro-structure part" or "a pro-structure." "A pro-structure part/pro-structure" of a protein can be herein interchangeably used with "a pro-part" of a protein. The signal peptide in a preproprotein or preprotein may be derived from the different protein or may be a signal peptide naturally occurring in the intended protein and it is preferably derived from a secretory protein of the host to be used. Alternatively, it may be modified to have the optimum codon depending on the codon usage of the host to be used.

[0020] Moreover, the signal peptide that can be used for the purpose of the invention may contain a part of the N-terminal amino acid sequence of a naturally

occurring mature protein from which the signal peptide is derived. A preproprotein can be especially called "a heterologously fused preproprotein" when the signal peptide is derived from the different protein. For example, when a protein is transglutaminase, they are referred to as "preprotransglutaminase," "protransglutaminase," and "heterologously fused preprotransglutaminase," respectively. A protein in which "the pro-part is cleaved" is referred to a protein wherein at least one or more amino acid that constitute its pro-part is removed by cleaving the peptide bond, including a protein having identical N-terminal amino acid with the naturally occurring protein and also includes a protein having one or more extra amino acids at the N-terminal deriving from the pro-part compared to the naturally occurring protein, and a protein having shorter amino acids sequence than that of a naturally occurring mature protein, provided that the protein has an activity of the intended protein.

[0021] As is described in the "Background of the Invention," a limited number of reports have been shown where the extracellular secretory production of a heterologous protein has been achieved using coryneform bacterium and the secretory production method have not been technically established. Also, it has not been known that a coryneform bacterium extracellularly secretes by itself a protein such as a protease. The known examples are endogenous DNase [US4965197] and the fact that the cell surface protein used in the present invention falls off from the cell surface to be found outside the cell [JP-Kokai No. 6-502548]. However, any signal peptide that involves in the secretion of a protein of coryneform bacterium has not been known except for the cell surface proteins. The only known cell surface proteins from coryneform bacterium, to date, are genes for PS1 and PS2, the cell surface proteins of *Corynebacterium glutamicum* [JP-Kokai No. 6-502548], and the gene for SlpA, the cell surface protein of *Corynebacterium ammoniagenes* (which may be abbreviated as C. *ammoniagenes* hereinafter) [JP-Kokai No. 10-108675]. Among these proteins, PS1 and SlpA share some homology (about 30%), but almost no homology was found among the others, and furthermore no homology was found in

the signal sequence domain any of the proteins. As the examples of signal sequences, the signal sequences of PS1 and PS2 from *Corynebacterium glutamicum* are shown in SEQ ID NO: 1 and SEQ ID NO:2, and the signal sequence of SlpA from *Corynebacterium ammoniagenes* is shown in SEQ ID NO: 3.

[0022] Therefore, the inventors cloned the gene of PS2 protein from *C. glutamicum* (formerly, *Brevibacterium lactofermentum*) ATCC13869 strain and determined the sequence. It was found that there were no differences in the signal sequence domain from the known sequence from *C. glutamicum*, but that there were two different amino acids in the sequence up to the N-terminal thirty-eighth amino acid residue of the mature cell surface protein (Asn for Thr residue at position 40 and Glu for Gly residue at position 55 in the amino acid sequence shown in SEQ ID NO: 5). The nucleotide sequence encoding sixty eight residues comprising thirty amino acid residues of the signal peptide and thirty eight amino acid residues from the N-terminal of the mature cell surface protein and its 5'-upstream region containing the promoter region is shown in SEQ ID NO: 4 and the amino acid sequence is shown in SEQ ID NO: 5.

[0023] Then, the inventor examined the secretion of a heterologous protein using the region containing the promoter region or the signal peptide region of the cell surface protein in order to determine whether the extracellular secretory production of a large amount of the heterologous protein can be achieved in a coryneform bacterium.

[0024] Since the transglutaminase gene from an actinomycete has a high GC content and the gene from a coryneform bacterium has a similar GC content to the gene from actinomycetes and also similar codon usage, there is an advantage that the gene from actinomycetes can be directly used. Therefore, the inventor investigated whether a transglutaminase gene from actinomycetes can be directly used or not, and found that the signal peptide of transglutaminase from actinomycetes did not successfully function in a coryneform bacterium. However, it is revealed that the transglutaminase gene encoding the mature protein containing the pro-structure part

from actinomycetes fused with the signal peptide of the cell surface protein from a coryneform bacterium effectively functioned without any modification and was efficiently secreted outside the cell as proprotein containing the pro-structure part. When the gene for transglutaminase with the pro-structure part which additionally comprises thirty amino acid residues from the cell surface protein and thirty eight amino acid residues from the N-terminal domain of the mature cell surface protein, for example, the gene for transglutaminase fused with the N-terminal domain of the mature cell surface protein was used, and the efficiency of the extracellular secretion of transglutaminase was further increased.

[0025] As used herein, a coryneform bacterium is an aerobic Gram-positive bacillus, which includes bacteria which were previously classified as *Brevibacterium* but currently unified as *Corynebacterium* (Int. J. Syst. Bacteriol., 41, 255(1981)) including Brevibacterium which is closely related to *Corynebacterium*. The use of *Corynebacterium* is advantageous in that it inherently secretes extremely less proteins outside the cell compared to molds, yeasts or bacteria belonging to *Bacillus* which have been previously recognized as suitable to effect the secretion of a heterologous protein, and which allow the purification process of the product to be easy and shortened when the secretory production of a heterologous protein is conducted. This is excellent in terms of its medium cost, the culturing procedure, and the yield, since it grows well on a simple culture medium such as those composed of ammonia, inorganic salts and so on.

[0026] Examples of *Corynebacterium* which can be used as a host bacterium in the present invention are mutants having the capacity of secreting heterologous proteins at least 2-fold higher than wild type *Corynebacterium glutamicum*. These mutants may be derived from wild type strains including *Brevibacterium saccharolyticum* ATCC14066, *Brevibacterium immariophilum* ATCC14068, *Brevibacterium lactofermentum* (*Corynebacterium glutamicum*) ATCC13869, *Brevibacterium roseum* ATCC13825, *Brevibacterium flavum* (*Corynebacterium glutamicum*) ATCC14067, *Corynebacterium acetoacidophilum* ATCC13870, *Corynebacterium glutamicum*

ATCC13032, *Corynebacterium lilium* (*Corynebacterium glutamicum*) ATCC15990, *Brevibacterium ammoniagenes* (*Corynebacterium ammoniagenes*) ATCC6871 or from the mutants thereof. The mutants of the present invention include mutant strains defective in the ability to produce glutamate, mutant strains for amino acids production such as lysine and the like, and mutant strains for producing other substances such as nucleic acids, for example, inosine. The mutants of the present invention may be obtained by selecting the strains having increased capacity of secretory production of proteins after UV-radiation or treating the bacteria with a chemical mutagen such as N-methyl-N'- nitrosoguanidine.

[0027] Particularly, *Corinebacterium glutamicum* (*C.glutamicum*) AJ12036 (FERM BP-734 - originally deposited on March 26, 1984 at presently, Independent Administrative Agency, National Institute of Advance Industrial Science and Technology, Tsukuba Central 6, 1-1, Higashi 1-Chome Tsukuba-shi, Ibaraki-ken, 305-8566 Japan) has the capacity of secretory production of heterologous proteins at least 2-3 fold higher than the parent strain (wild type strain) under the optimum culture condition as measured as the amount of accumulation, which may be due to the mutation in functional genes responsible for the secretion of proteins. Thus, this strain is suitable as a host. Furthermore, it is particularly preferable to use a strain which is derived from such a mutant and which is modified such that it does not produce cellular surface proteins, because the purification of secreted heterologous proteins becomes easier. Such modifications may be conducted by introducing a mutation into the regions encoding the cellular surface proteins or their expression control regions existing on the genome by mutagenesis or using gene recombination techniques.

[0028] The genetic construct which can be used in the present invention generally includes a promoter, a sequence encoding a proper signal peptide and a nucleic acid fraction encoding an intended protein, and a regulatory sequence (an operator or terminator, etc.) necessary to express the gene for the intended protein in a coryneform bacterium, at a proper position such that they can function. The intended

protein may have a pro-structure part at the N-terminal. Vectors that may be used for this construct are not particularly limited and include any vector which can function in a coryneform bacterium, and may be those which autonomously multiply, such as plasmids or vectors which are integrated into the chromosome of the bacterium. Plasmids derived from coryneform bacteria are particularly preferable. These include, for example, pHM1519 (Agric. Biol. Chem., 48, 2901-2903(1984)), pAM330 (Agric. Biol. Chem., 48, 2901-2903(1984)), and plasmids obtained by modifying them to possess drug-resistant genes.

[0029] Artificial transposons and the like may also be used. When a transposon is used, the intended gene is introduced into the chromosome through homologous recombination or by its own transposing ability.

[0030] Promoters which can be used in the invention are not particularly limited. Any promoter which can function in the cell of a coryneform bacterium may be generally used. It may also be a promoter derived from a different species, for example, a promoter derived from *E. coli*, such as tac promoter, etc.. Among these promoters, a potent promoter is more preferable, including tac promoter, etc..

[0031] Examples of promoters derived from a coryneform bacterium include promoters of the genes of cell surface proteins PS1, PS2 and SlpA, promoters of the genes in biosynthetic systems of different amino acids, for example, glutamate dehydrogenase gene in the glutamic acid biosynthetic system, glutamine synthetase gene in the glutamine synthetic system, aspartokinase gene in the lysine biosynthetic system, homoserine dehydrogenase gene in the threonine biosynthetic system, acetohydroxylate synthase gene in the isoleucine and valine biosynthetic system, 2-isopropylmalate synthase gene, glutamate kinase gene in the proline and arginine synthetic system, phosphoribosyl-ATP pyrophosphorylase gene in the histidine biosynthetic synthesis, deoxyarabinohepturonic acid phosphate(DAHP) synthase gene in the aromatic amino acid biosynthetic system such as tryptophan, tyrosine and phenylalanine, etc., phosphoribosyl pyrophosphate (PRPP) amidotransferase gene, inosinate dehydrogenase gene and guanylate synthase gene in the nucleic acid

biosynthetic system such as inosinate and guanylate.

[0032] The signal peptide which is used in the present invention is the signal peptide of a secretory protein from the host, Coryneform bacterium, and preferably it is the signal peptide of a cell surface protein from a Coryneform bacterium. Cell surface proteins include PS1 and PS2 derived from *C. glutamicum* (JP-Kokai No. 6-502548), and SlpA derived from *C. Ammoniagenes* (JP-Kokai No. 10-108675). The amino acid sequence of PS1 is shown in SEQ ID NO:2, the amino acid sequence of PS2 in SEQ ID NO:1 and the amino acid sequence of SlpA is shown in SEQ ID NO:3. Additionally, it is reported that DNase from a coryneform bacterium also has a signal peptide, as described in US Patent No. 4965197, which may also be used in the present invention.

[0033] A portion of N-terminal amino acid sequence of the secretory protein from which the signal peptide derives may be connected to the signal peptide. The signal sequence is cleaved by a signalpeptidase during the extracellular secretion of the translated product. In addition, the gene encoding the signal peptide can be used either in native form or in modified form containing the optimum codons depending on the codon usage in the host to be used.

[0034] When these signal peptides are used, the genes encoding intended proteins are connected to the 3'-terminal of the genes encoding the signal peptides and are located such that they are subject to the regulation of expression by the promoters described above.

[0035] The useful proteins which can be secreto-produced according to the present invention essentially include, but are not limited to, all of the secretory proteins derived from animals and plants and microorganisms. For example, proteins such as a protease, an exopeptidase, an aminopeptidase, a carboxypeptidase, a collagenase and a chitinase can be secreto-produced according to the present invention. Proteins which are prepared by the secretory production according to the present invention are preferably naturally occurring secretory proteins, more preferably proteins having additional pro-structure parts. Transglutaminase is particularly preferred as a useful

protein prepared by the secretory production according to the present invention. As transglutaminase genes, for example, genes for a transglutaminase of secretion type derived from actinomycetes, for example, *S. mobaraense* IFO 13819, *S. cinnamoneum* IFO 12852, *Streptoverticillium griseocarneum* IFO 12776, *Streptomyces lydicus* [WO9606931], etc. and molds such as Oomyceted [WO9622366], etc can be used for the purpose of the present invention. The genes encoding these proteins can be modified depending on the type of the host to be used and in order to achieve the desired activity, and may comprise the addition, deletion, replacement of one or more amino acid residues, and optionally may be converted to contain the optimum codon depending on the frequency of codon usage in the host.

[0036] When the protein produced by the secretory production according to the present invention is the protein naturally expressed as a prepropeptide, it is preferable to use the gene fragment encoding the proprotein containing the pro-structure part (pro-part), although it is not essential. When the gene encoding a preproprotein is used, the pro-part of the protein obtained as the result of the expression of the gene may be cleaved by any appropriate means, for example, by a protease. For this, aminopeptidases, endopeptidases which can cleave at an appropriate site, or more specific proteases may be used. It is preferable to use the proteases which cleave the protein such that the cleaved protein has an equivalent activity or higher activity than that of the naturally occurring protein. Alternately the gene sequence encoding the intended protein or encoding the pro-structure part of the intended protein can also be modified and designed to express the protein having the recognition site for protease specific to the desired location. General molecular biotechnological procedures including such modification techniques, gene cloning techniques and detection techniques for the produced proteins are well known to those skilled in the art and reference can be made to Sambrook et al., 1989, Molecular Cloning: A Laboratory Manual, Second Edition (1989) Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York; DNA Cloning: A Practical Approach, Volumes I and II (D. N. Glover ed. 1985); F.M. Ausubel et al.(Eds), Current Protocols in Molecular Biology,

John Wiley & Sons, Inc. (1994); PCR Technology: Principles and Application for DNA Amplification, H. Erlich, ed., Stockton Press and etc..

[0037] The N-terminal region of the protein which may be finally obtained according to the present invention is not necessarily identical to that of the naturally occurring protein and therefore, one to several amino acids may be further added to or deleted from the naturally occurring protein. When a protease is used, it is preferred that the produced protein is cleaved at about the same position as that of the naturally occurring protein in terms of its activity and it is more preferable that it is identical to the mature peptide of a naturally occurring protein. Therefore the specific proteases which cleave the propeptide at the position such that it generates the same protein as the naturally occurring mature protein are generally most preferable. However, for a particular purpose, the peptides having longer or shorter sequence of amino acid residue by one to several residues at the N-terminal compared to the N-terminal of the naturally occurring protein may possess more appropriate activity. Such proteases include, for example, Dispase (available from Boehringer Manheim Co.) which is commercially available and proteases obtained from the culture medium of microorganisms, such as, for example, the culture medium of actinomycetes. Such proteases may be used in an unpurified state or optionally may be used after being purified to the appropriate purity.

[0038] Other examples of suitable proteases for removing the pro-part of protransglutaminases derived from Streptomyces is SAMP45, a serine protease produced by *Streptomyces albogriseolus* (hereinafter it may be abbreviated as *S. albogriseolus*). The gene sequence and encoded full length amino acid sequence (1-13: signal sequence, 32-76: pro-part, 77-407: mature transglutaminase) for *S. mobaraense* transglutaminase is shown in SEQ ID:6 and SEQ ID:7, respectively. In the case of *S. mobaraense* protransglutaminase, since SAMP45 cleaves between Ser^{72} and Phe^{73} in the pro-structure part, the resulting protein has the structure where an additional 4 amino acids (Phe-Arg-Ala-Pro, SEQ ID:60) deriving from the C-terminal of pro-part attached to the N-terminal of the naturally occurring mature

transglutaminase. The inventors confirmed that such proteins have the transglutaminase activity. The sequence of SAMP45 gene has already been determined and the amino acid sequence of the protein with the additional pro-structure part (proSAMP45) is shown in SEQ ID NO:8 (J. Bacteriol., 179, 430-438 (1997)), as well.

[0039] Additionally, mature transglutaminase identical to the naturally occurring transglutaminase can be obtained by using the proline-specific peptidase produced by *S. mobaraense* (svPEP), which has been found by the inventors, together with SAMP45, which results in the removal of the four amino acids of Phe-Arg-Ala-Pro added at the N-terminal.

[0040] This svPEP is an enzyme that cleaves specifically the peptides or the peptide analogues represented by the following formula(I) at the site shown with * in the formula, that is, at the carboxyl terminal side of the third or fourth proline residue from the N-terminal:

Y-Pro-*-Z (I)

wherein Y represents an oligopeptide consisting of two or three amino acid residues and Z represents an amino acid, peptide, amide or ester.

[0041] The nucleotide sequence of svPEP gene and the entire encoded amino acid sequence is shown in SEQ ID:9 and SEQ ID:10, respectively. When svPEP is reacted on the protransglutaminase together with a protease in the form of broth of *S. mobaraense* or *S. mobaraense* cells, the pro-structure part can be cleaved completely, resulting in the mature transglutaminase from which the pro-structure part is completely removed. Alternatively, the mature transglutaminase of which pro-structure part is completely removed can be similarly obtained by culturing a coryneform bacterium where pre-pro svPEP gene together with a protease gene are introduced into a coryneform bacterium which releases a protransglutaminase extracellularly by secretory production. Moreover a mature transglutaminase having the same structure as that of a naturally occurring form can be efficiently produced by introducing similarly both SAMP45 gene and svPEP gene into a coryneform

bacterium to which pre-protransglutaminase gene has been introduced, and by allowing the bacterium to secreto-produce protransglutaminase and SAM45 as well as svPEP extracellularly or at the surface of the cells.

[0042] The method for introducing the genetic constructs that can be used in the present invention into a coryneform bacterium is not limited to particular methods and the methods generally used include, for example, the protoplast method (Gene, 39, 281-286 (1985)), the electroporation method (Bio/Technology, 7, 1067-1070) (1989)), etc.. The resulting transformant can be cultured according to the conventional methods and conditions. For example, the transformant can be cultured with a conventional medium containing carbon sources, nitrogen sources and inorganic sources. Trace amount of organic nutrients such as vitamins and amino acids can be optionally added to the medium in order to achieve the growth to greater extent.

[0043] Carbohydrates such as glucose and sucrose can be used as carbon sources, and organic acids such as acetic acid, alcohols and others can be used. Gaseous ammonia, aqueous ammonia, ammonium salt and others can be used as nitrogen sources. As inorganic ions, calcium ion, magnesium ion, phosphorus ion, potassium ion, ferrous or ferric ion and others are optionally used as necessary. The culture may be conducted for about 1 to 7 days under the aerobic condition in the appropriate range of pH between 5.0 and 8.5 and of the temperature between 15°C and 37°C. By culturing the transformant under such conditions, a large amount of an intended protein is produced intracellularly and is efficiently secreted extracellularly. Transglutaminase is generally known to be lethal when it is largely accumulated in the cells of microorganisms, but according to the present invention, transglutaminase is continuously produced without generating lethal effects, because the intracellularly produced transglutaminase is released extracellularly.

[0044] The proteins which have been secreted in the medium according to the present invention can be isolated and purified from incubated culture medium according to methods well known to those skilled in the art. For example, the proteins can be isolated and purified by removing the cells from the medium by

centrifugation, etc., and then by using known appropriate methods such as salting-out, ethanol precipitation, ultrafiltration, gel filtration chromatography, ion-exchange column chromatography, affinity chromatography, medium high-pressure liquid chromatography, reversed-phase chromatography, hydrophobic chromatography or the combination thereof. The proteins secreted at the surface of the cells according to the present invention can be isolated and purified by using methods well known to those skilled in the art, for example, by solubilizing them with increased salt concentrations or surfactants, and then using similar methods as those used for proteins secreted in the medium. Additionally, in some cases, the proteins secreted at the surface of the cell may be used without solubilization, for example, as immobilized enzymes.

Examples

[0045] The present invention will be illustrated by the following examples, but these examples should not be construed as limiting as to the scope of the present invention.

[0046] Example 1 : Expression of prepro-transglutaminase derived from *S. mobaraense* IFO13819 in *C. glutamicum* ATCC13869

(1) Acquisition of the transglutaminase gene derived from *S. mobaraense* IFO13819

[0047] The sequence of transglutaminase gene derived from *S. mobaraense* DSMZ strain has already been determined [Eur. J. Biochem., 257, 570-576(1998)]. The primers shown in SEQ ID NO: 11 and SEQ ID NO: 12 were synthesized with reference to the sequence and the region encoding the sequence of mature transglutaminase was amplified using PCR method with the chromosomal DNA of *S. mobaraense* IFO13819 prepared according to the conventional procedure (the method of Saito and Miura [Biochim, Biophys. Acta, 72, 619(1963)]. For PCR reaction, Pyrobest DNA polymerase(Takarashuzo Co. Ltd.) was used and the reaction condition followed the protocol recommended by the manufacturer.

(SEQ ID NO: 11) 5'-GACTCCGACGACAGGGTCACCCCTCCCGCC-3'

(SEQ ID NO: 12) 5'-CGCTCACATCACGGCCAGCCCTGCTTTACC-3'

SEQ ID NO: 11 and SEQ ID NO: 12 : PCR primer

[0048] The DNA probe was then generated by conducting the reaction using amplified DNA fragment of about 1.0 kb with [α-^{32}P]dCTP and Random Primer DNA Labeling Kit Ver. 2 (Takarashuzo Co. Ltd.) according the protocol attached to the Kit. It was confirmed that the transglutaminase gene was present in the fragment of about 4 kb excised with restriction enzyme Sac I by Southern blot hybridization using the generated probe and the chromosomal DNA of *S. mobaraense* IFO13819 according to the conventional method, as described in Molecular Cloning 2nd edition J. Sambrook, E. F. Fritsch and T. Maniatis, Cold Spring Harbor Laboratory Press, p9. 31 (1989). Accordingly, the fragment of about 4 kb which had been generated by SacI digestion of the chromosomal DNA of *S. mobaraense* IFO13819 was recovered through agarose gel electrophoresis using EASYTRAP Ver. 2 (Takarashuzo Co. Ltd.) and was inserted into Sac I site of pUC18 (Takarashuzo Co. Ltd.) which was introduced into competent cells of *Escherichia coli* JM109 (Takarashuzo Co. Ltd.) to generate a library.

[0049] The bacterium strain harboring the plasmid where the transglutaminase gene fragment was cloned was obtained by screening the library using the previously generated DNA probe for transglutaminase by colony hybridization as described in Molecular Cloning 2nd edition J. Sambrook, E. F. Fritsch and T. Maniatis, Cold Spring Harbor Laboratory Press, p1. 90(1989). The plasmid was recovered from this strain and designated as pUITG. The sequence of the fragment cloned in pUITG was determined, which confirmed that the transglutaminase gene from *S. mobaraense* IFO13819 had the same nucleotide sequence as that of the transglutaminase from *S. mobaraense* DSMZ strain.

[0050] The determination of the nucleotide sequence revealed that the SacI fragment of about 4kb was the incomplete DNA fragment from which the signal sequence (the pre-part) was partially deleted. Accordingly, the cloning of the

promoter region and the entire signal sequence region was attempted. The cloning was performed using TAKARA LA PCR in vitro Cloning kit (Takarashuzo Co. Ltd.) and the synthesized primers shown in SEQ ID NO: 13 and SEQ ID NO:14 according to the attached protocol.

(SEQ ID NO: 13) 5'-GTGACCCTGTCGTCGGAGTC-3'

(SEQ ID NO: 14) 5'-GGCATCCTGTCGAGCGGCTC-3'

SEQ ID NO: 13 and SEQ ID NO: 14: PCR primers for the promoter region and the signal sequence of *S. mobaraense*

[0051] Consequently when a cassette primer of SalI was used, the PCR-amplified fragment of about 800 bp was obtained and the sequencing of the fragment confirmed that the fragment contained the promoter region of the transglutaminase gene and the signal sequence region. Accordingly, the PCR-amplified fragment of about 800 bp was inserted into SmaI site of pVC7 described in JP-Kokai No. 9-070291 to obtain pVITGS5. Additionally plasmid pUITG was digested with SacI, the fragment of about 4kb was recovered through agarose electrophoresis, and the fragment was inserted to SacI site of pVITGS5 to construct plasmid pVITGC which contained the full-length transglutaminase gene. The determination of the nucleotide sequence was performed using Dye Terminator Cycle Sequencing kit (PE Applied Biosystems) and DNA Sequencer 373A (PE Applied Biosystems). The sequence of the preprotransglutaminase gene is shown in SEQ ID NO: 6. It was supposed that the N-terminal 31 amino acids sequence (No. 1-31) was the signal sequence (the pre-part), the N-terminal 45 amino acids sequence (No.32-76) was the pro-part and the N-terminal 331 amino acids sequence (No.77-407) was the mature transglutaminase.

(2) Conversion of the promoter region of transglutaminase gene

[0052] The sequence of the gene for PS2 which is a surface protein of C. glutamicum has already been determined [Mol. Microbiol., 9, 97-109(1993)]. Primers shown as SEQ ID NO: 15 and SEQ ID NO:16 were synthesized on referring to that sequence, and the region which contains the promoter located at the 5'-

upstream region of the initiation codon of PS2 protein gene was amplified using PCR method from the chromosomal DNA of *C. glutamicum* ATCC13869 prepared according to a conventional method.

(SEQ ID NO: 15)

5'-AAATTCCTGTGAATTAGCTGATTTAG-3'

(SEQ ID NO: 16)

5'-GAGCTCTCCGGCGTATGCGCATAGAGGCGAAGGCTCCTTGAATA-3'

SEQ ID NO: 15 and SEQ ID NO:16: PCR primers

[0053] On the other hand, the primers shown in SEQ ID NO: 12 and SEQ ID NO: 17 were synthesized based on the sequence of the transglutaminase gene determined in Example 1(1), and the region of the preprotransglutaminase gene was amplified using PCR method from pUITG obtained in Example 1(1).

(SEQ ID NO: 12) 5'-CGCTCACATCACGGCCAGCCCTGCTTTACC-3'

(SEQ ID NO: 17) 5'-ATGCGCATACGCCGGAGAGCTCTCGTCTTC-3'

SEQ ID NOs: 12 and 17: PCR primer

[0054] Then, the fusion gene of transglutaminase fused with the additional pre-pro structure part, which was ligated to the region comprising the promoter of the cell surface protein gene from C. glutamicum ATCC13869, was amplified by performing cross-over PCR with SEQ ID NO: 15 and SEQ ID NO:12 using the mixture of 1 μl each of the PCR solution of the amplified region comprising the promoter of PS2 gene of *C. glutamicum* ATCC13869 and of the amplified pre-protransglutaminase gene region, as the templates. The amplified fragment of about 1.8 kb was detected by agarose gel electrophoresis. This fragment was recovered from the agarose gel with EASYTRAP Ver. 2 (Takarashuzo Co. Ltd.) and inserted into SmaI site of pVC7 as described in JP-Kokai No. 9-070291 to obtain pVKPTG0. The nucleotide sequence of the inserted fragment was determined according to the method described above and it was confirmed that the fusion gene was constructed as expected.

(3) Expression of the pre-protransglutaminase gene in *C. glutamicum* ATCC13869

[0055] *C. glutamicum* ATCC13869 was transformed with the pVITGC constructed in Example 1(1) (both the promoter and the pre-protransglutaminase gene were derived from *S. mobaraense*) or with the pVKPTGO constructed in Example 1(2) (the promoter was derived from PS2 gene of *C. glutamicum* ATCC13869 and the pre-protransglutaminase gene was derived from *S. mobaraense*) and the strains grown on the CM2S agar medium comprising 5 mg/l of chloramphenicol (10g of yeast extract, 10g of tryptone, 5g of sucrose, 5g of NaCl, 5g of agar per liter of distilled water) were selected. The selected *C. glutamicum* ATCC13869 cells harboring pVITGC or pVKPTGO were cultured in MM culture medium (30g of glucose, 0.4g of magnesium sulfate heptahydrate, 30g of ammonium sulfate, 1g of potassium dihydrogenphosphate, 0.01g of ferrous sulfate heptahydrate, 0.01g of manganese(II) sulfate pentahydrate, 200μg of thiamine hydrochloride, 500μg of biotin, 0.15g of DL-methionine, 50g of calcium carbonate per liter of distilled water, adjusted to pH 7.5) comprising 5 mg/l of chloramphenicol at 30 °C for 48 hours, respectively. After the incubation was finished, 10 μl of the supernatant of the culture was subjected to SDS-PAGE and then to Western blot using anti-transglutaminase antibody as described in Biosci. Biotechnol. Biochem., 58, 82-87(1994) according to the conventional method (for example, the general procedure as described in J. Sambrook et al. (1989)(supra)).

[0056] Consequently, the secretion of transglutaminase could not be detected. From the above results, it was confirmed that the signal sequence of transglutaminase from *S. mobaraense* did not function in *C. glutamicum* ATCC13869.

[0057] Example 2: Secretory production of mature transglutaminase using the fusion gene encoding the signal peptide of the cell surface protein of *Corynebacterium glutamicum* (*C. glutamicum* ATCC13869) and the mature transglutaminase derived from S. *mobaraense* IFO13819

(1) Construction of the transglutaminase gene containing the signal sequence of cell surface protein of *C. glutamicum* ATCC13869

[0058] The sequence of the gene of PS2 which is the cell surface protein of *C.*

glutamicum has been already determined [Mol. Microbiol., 9, 97-109(1993)]. The primers shown as SEQ ID NO:15 and SEQ ID NO:18 were synthesized on referring to the sequence, and the region encoding the N-terminal 44 amino acid residues (30 amino acid residues of the signal peptide and 14 amino acid residues of the mature cell surface protein) of the protein corresponding to PS2 and 5'-upstream region containing the promoter region were amplified using PCR method with the chromosomal DNA of *C. glutamicum* ATCC13869 prepared according to the method described in Example 1(2). The primer shown in SEQ ID NO:18 also comprises the sequence encoding the amino acid sequence from the N-terminal region of the mature transglutaminase in order to construct the fusion gene fused with transglutaminase.

(SEQ ID NO: 15)

5'- AAATTCCTGTGAATTAGCTGATTTAG-3'

(SEQ ID NO:18)

5'-GGGGTGACCCTGTCGTCGGAGTCGTTGAAGCCGTTGTTGATGTTGAA-3'

SEQ ID NOs:15 and 18: PCR primer

[0059] On the other hand, primers shown in SEQ ID NO:11 and SEQ ID NO: 12 were synthesized based on the sequence of the transglutaminase gene determined in Example 1(1) and the region of mature transglutaminase gene was amplified using PCR method with pUITG obtained in Example 1(1).

[0060] The fusion gene of the mature transglutaminase, which was connected to the region encoding the N-terminal 44 amino acid residues of *C. glutamicum* ATCC13869 and to the 5'-upstream region comprising the promoter gene of the cell surface protein gene, was amplified by performing cross-over PCR with SEQ ID NO:15 and SEQ ID NO:12 using the mixture of 1 μl of PCR solution of the amplified region encoding the N-terminal 44 amino acid residues of the protein corresponding to PS2 of C. glutamicum and of the amplified 5'-upstream region containing the promoter, and 1 μl of PCR solution of the amplified mature transglutaminase gene region, as the templates.

[0061] The amplified fragment of about 1.7 kb was detected by agarose

electrophoresis. This fragment was recovered from the agarose gel using EASYTRAP Ver. 2 (Takarashuzo Co. Ltd.) and inserted into SmaI site of the pVC7 described in JP-Kokai No. 9-070291 to obtain pVKPTG3. The nucleotide sequence of the inserted fragment was determined according to the method described above and it was confirmed that the expected fusion gene was constructed.

[0062] Additionally, the fusion mature transglutaminase gene of about 1.7 kb, which had been ligated to the region encoding the N-terminal 44 amino acid residues from *C. glutamicum* ATCC13869 and the 5'-upstream region comprising the promoter of the cell surface protein gene, was excised by digesting pVKTG3 with KpnI and XbaI and recovered using agarose electrophoresis. This fragment was inserted into the KpnI-XbaI site of pPK4 described in JP-Kokai No. 9-322774 to construct pPKTG3.

(2) Secretion of mature transglutaminase using the signal sequence of the cell surface protein of *C. glutamicum* ATCC13869

[0063] *C. glutamicum* ATCC13869 was transformed with the constructed plasmid pVKTG3 or pPKTG3 (in both cases the gene comprising the promoter and the gene encoding signal peptide and the N-terminal 14 amino acid residues were derived from *C. glutamicum* ATCC13869, and the mature transglutaminase gene was derived from *S. mobaraense*) and the strains grown on the CM2S agar medium comprising 5 mg/l of chloramphenicol or 25 mg/l of kanamycin and were selected. The selected *C. glutamicum* ATCC13869 cells containing pVITG3 or pVKPTG3 were then cultured in liquid MM culture medium, described above, comprising 5 mg/l of chloramphenicol or 25 mg/l of kanamycin at 30°C for 48 hours, respectively. After the incubation was finished, 10 μl of the supernatant of the culture was subjected to SDS-PAGE and then Western blot was performed according to a conventional method using anti-transglutaminase antibody as described in Biosci. Biotechnol. Biochem., 58, 82-87(1994). As a result, a small amount of secreted transglutaminase having the similar molecular weight to that of the mature

transglutaminase could be detected in the supernatant of the culture of both strains.

[0064] Example 3: Secretory production of pro-transglutaminase using pro-transglutaminase fusion gene (heterologously fused prepro-transglutaminase fusion gene) derived from *S. mobaraense* IFO13819 ligated to the signal peptide of cell surface protein of *C. glutamicum* ATCC13869

(1) Construction of transglutaminase gene (heterologously fused prepro-transglutaminase fusion gene) containing the additional pro-structure part with the signal peptide of cell surface protein of *C. glutamicum* ATCC13869.

[0065] The primers shown in SEQ ID NO:19, SEQ ID NO:20, SEQ ID NO:21 and SEQ ID NO:22 were synthesized on referring to the sequence of the gene of PS2 which was the cell surface protein of *C. glutamicum* [Mol. Microbiol., 9, 97-109(993)]. The coding region for the N-terminal 30, 31, 44 or 68 amino acid residues (the region comprising 30 amino acid residues of the signal peptide) and the 5'-upstream region containing the promoter region of the protein corresponding to PS2 were amplified respectively by PCR method using the combination of SEQ ID NO: 15 and SEQ ID NO: 19, or of SEQ ID NO: 15 and SEQ ID NO:20, or of SEQ ID NO: 15 and SEQ ID NO: 21, or of SEQ ID NO:15 and SEQ ID NO:22 from the chromosomal DNA of *C. glutamicum* ATCC13869 prepared according to the method described in Example 1(2).

[0066] Primers shown in SEQ ID NO:19, SEQ ID NO:20, SEQ ID NO:21 and SEQ ID NO: 22 comprise the sequences encoding the N-terminal amino acids of pro-transglutaminase in order to construct the fusion gene fused with the transglutaminase having the pro-structure part.

(SEQ ID NO:15) 5'-AAATTCCTGTGAATTAGCTGATTTAG-3'

(SEQ ID NO: 19)

5'-

TTCGTCTCTTCCCCCGCGCCATTGTCAGCGAATGCTGGGATAGCAACGCC-

3'

(SEQ ID NO: 20)

5'-

CTTCGTCTCTTCCCCCGCGCCATTGTCCTGAGCGAATGCTGGGATAGCTAC-

3'

(SEQ ID NO: 21)

5'-

CTTCGTCTCTTCCCCCGCGCCATTGTCGTTGAAGCCGTTGTTGATGTTGAA-

3'

(SEQ ID NO: 22)

5'-

CTTCGTCTCTTCCCCCGCGCCATTGTCAGTCAGGTCGCGGAGGGTTTCCTC-

3'

SEQ ID NOs:15, 19, 20, 21 and 22: PCR primers

[0067] On the other hand, the primers shown in SEQ ID NO:23 and SEQ ID NO:12 were synthesized based on the sequence of the transglutaminase gene determined in Example 1(1) and the pro-transglutaminase gene region was amplified using PCR method with pUITG obtained in Example 1(1).

(SEQ ID NO:12) 5'-CGCTCACATCACGGCCAGCCCTGCTTTACC-3'

(SEQ ID NO:23) 5'-GACAATGGCGCGGGGGAAGAGACGAAGTCC-3'

SEQ ID NOs: 12 and 23 : PCR primer

[0068] Then the heterologously fused pro-transglutaminase gene ligated to the respective region encoding its N-terminal 30, 31, 44 or 68 amino acid residues and the 5'-upstream region comprising the promoter region of the protein gene corresponding to PS2 from *C. glutamicum* ATCC13869, that is, the fragments of heterologously fused prepro-transglutaminase genes which were ligated to the promoter of the gene of the cell surface protein of *C. glutamicum* ATCC13869, was amplified by performing cross-over PCR with SEQ ID NO: 15 and SEQ ID NO:12 using the mixture which comprises 1 μl of PCR solution of the 5'-upstream region containing the promoter region of the gene of the protein corresponding to PS2 of *C. glutamicum*

ATCC13869 and one of the amplified region encoding N-terminal 30, 31, 44 or 68 amino acid residues of the protein, and 1 μl of PCR solution of the amplified region of the gene for the transglutaminase having the pro-structure part, as the templates.

[0069] The amplified fragments ranging about 1.8 kb to 1.9 kb was detected by agarose electrophoresis. These fragments were recovered from the agarose gels with EASYTRAP Ver. 2 (Takarashuzo Co. Ltd.) and inserted into SmaI site of pVC7 as described in JP-Kokai No. 9-070291 to obtain pVKPTG1, pVKPTG2, pVKPTG3 and pVKPTG4, respectively. The nucleotide sequences of the inserted fragments were determined according to the aforementioned method and it was confirmed that the expected fusion genes were expected.

[0070] Additionally, the fusion genes of about 1.8 kb to 1.9 kb of transglutaminase having the pro-structure parts, which was ligated to the respective region encoding the 30, 31, 44 or 68 amino acid residues and the 5'-upstream region comprising the promoter region of the gene of the protein corresponding to PS2 of *C. glutamicum*, were excised by digesting pVKPTG1, pVKPTG2, pVKPTG3 or pVKPTG4 respectively with KpnI and XbaI and were recovered by agarose electrophoresis. These fragments were inserted into KpnI-XbaI site of pPK4 described in JP-Kokai No. 9-322774 to construct pPKPTG1, pPKPTG2, pPKPTG3 and pPKPTG4.

(2) Secretion of pro-transglutaminase using the signal sequence of the cell surface protein of *C. glutamicum* ATCC13869

[0071] *C. glutamicum* ATCC13869 was transformed with the constructed plasmid pVKPTG1, pVKPTG2, pVKPTG3, pVKPTG4, pPKPTG1, pPKPTG2, pPKPTG3 or pPKPTG4 and the strains grown on the CM2S agar medium, described above, comprising 5 mg/l of chloramphenicol or 25 mg/l of kanamycin and selected. The selected *C. glutamicum* ATCC13869 harboring pVKPTG1, pVKPTG2, pVKPTG3, pVKPTG4, pPKPTG1, pPKPTG2, pPKPTG3 or pPKPTG4 were then cultured in MM culture medium, described above, comprising 5 mg/l of chloramphenicol or 25 mg/l of kanamycin at 30°C for 48 hours, respectively. After the incubation was finished,

10 μl of the supernatant of the culture was subjected to SDS-PAGE and then Western blot was performed using anti-transglutaminase antibody as described in Biosci. Biotechnol. Biochem., 58, 82-87(1994) according to the conventional method. As a result, the secretion of the similar amount of transglutaminase having the pro-structure part was confirmed for both of the vectors, pVC7 or pPK4, and the significant differences in the secreted amount were observed depending on the length of N-terminal amino acid residues of the mature form of the protein corresponding to PS2. The representative secreted amounts are shown in Table 1.

Table 1. The secreted amount of pro-transglutaminase using the signal sequence of the cell surface protein of *C. glutamicum* ATCC13869

plasmid	pro-transglutaminase (mg/l)
pPKPTG1	78
pPKPTG4	210

[0072] Example 4: Secretory production of pro-transglutaminase using the fusion gene having the sequence encoding the signal sequence of the cell surface protein of *C. ammoniagenes* and the pro-transglutaminase derived from *S. mobaraense* IFO13819

(1) Construction of the transglutaminase gene having the additional pro-structure part and the signal sequence of the cell surface protein of *C. ammoniagenes* (heterologously fused preprotransglutaminase fusion gene)

[0073] The primers shown in SEQ ID NO:24 and SEQ ID NO:25 were synthesized on referring to the sequence of the gene of the cell surface protein (SlpA) [JP-Kokai No. 10-108675] of *C. ammoniagenes* and the region comprising the 5'-upstream region containing the promoter region of the cell surface protein (SlpA) gene and the region encoding the N-terminal 25 amino acid residues (the signal peptide) of SlpA was amplified using PCR method from the chromosomal DNA of *C. ammoniagenes* prepared according to the conventional method. The primer shown in SEQ ID NO:25 also comprises the sequence encoding the N-terminal amino acids of the pro-

transglutaminase in order to construct the fusion gene fused with the pro-transglutaminase.

(SEQ ID NO:24) 5'-GCCCAGAAGCCCAAAATTGAGATTT-3'

(SEQ ID NO:25)

5'-

CTTCGTCTCTTCCCCCGCGCCATTGTCTGCCGTTGCCACAGGTGCGGCCAG

C-3'

SEQ ID NOs: 24 and 25: PCR primers

[0074] The fusion transglutaminase gene containing the additional pro-structure part which was ligated to the region encoding the N-terminal 25 amino acid residues of *C. ammoniagenes* and the 5'-upstream region comprising the promoter region of the cell surface protein (SlpA) gene (heterologously fused prepro-transglutaminase gene) was amplified by performing cross-over PCR with SEQ ID NO:24 and SEQ ID NO:12 using the mixture, as the templates, containing 1 μl of PCR solution of the amplified 5'-upstream region containing the promoter region of the gene of the cell surface protein (SlpA) and the region encoding the N-terminal 25 amino acid residues of the cell surface protein (SlpA) of *C. ammoniagenes* and 1 μl of PCR solution of the region of the gene for the transglutaminase having the additional pro-structure part which had been amplified in Example 3(1). The amplified fragment of about 1.7 kb was detected by agarose electrophoresis. This fragment was recovered from agarose gel using EASYTRAP Ver. 2 (Takarashuzo Co. Ltd.) and was inserted into SmaI site of pVC7 to obtain pVSPTG1.

(2) Conversion of the promoter region: Ligation with the promoter of the cell surface protein gene of *C. glutamicum* ATCC13869

[0075] The primers shown in SEQ ID NO:15 and SEQ ID NO:26 were synthesized on referring to the sequence of the gene of PS2 which is the cell surface protein [Mol. Microbiol., 9, 97-109(1993)] of *C. glutamicum*. The 5'-upstream region comprising the promoter region of the gene for the protein corresponding to PS2 was amplified

using PCR method from the chromosomal DNA of *C. glutamicum* ATCC13869 prepared according to the method in Example 1(2). The primer shown in SEQ ID NO:26 also comprises the sequence encoding the N-terminal amino acids of the signal sequence of the cell surface protein (SlpA) of *C. ammoniagenes* in order to construct the fusion gene with transglutaminase gene having the pro-structure part connected to the signal sequence of the cell surface protein (SlpA) of *C. ammoniagenes* (heterologously fused prepro-transglutaminase fusion gene).

(SEQ ID NO:15) 5'-AAATTCCTGTGAATTAGCTGATTTAG-3'

(SEQ ID NO: 26)

5'-

CGCAGCCAGCGATTTCATGCGTTTCATAGAGGCGAAGGCTCCTTGAATAG

GT-3'

SEQ ID NO:15, 26: PCR primer

[0076] On the other hand, the primers shown in SEQ ID NO:27 and SEQ ID NO:12 were synthesized based on the sequence of the transglutaminase fusion gene having the additional pro-structure part and the signal sequence of the cell surface protein (SlpA) of *C. ammoniagenes*. The region of the transglutaminase having the additional pro-structure part was then amplified by PCR method from pVSPTG1 obtained in Example 4(1) with the primers.

(SEQ ID NO: 12) 5'-CGCTCACATCACGGCCAGCCCTGCTTTACC-3'

(SEQ ID NO:27) 5'-ATGAAACGCATGAAATCGCTGGCTGCGGCG-3'

SEQ ID NOs: 12 and 27: PCR primer

[0077] The fusion gene of transglutaminase having the pro-structure part, which was ligated to the region encoding the N-terminal 25 amino acid residues of the cell surface protein (SlpA) of *C. ammoniagenes* and to the 5'-upstream region containing the promoter region of the gene of the protein corresponding to PS2 of *C. glutamicum* ATCC13869, was then amplified by performing cross-over PCR with SEQ ID NO:15 and SEQ ID NO:12 using the mixture comprising 1 μl of PCR solution of the amplified 5'-upstream region containing the promoter region of the gene for the

protein corresponding to PS2 of *C. glutamicum* and 1 μl of PCR solution of the amplified region of the gene for the transglutaminase having the pro-structure part which had the signal sequence of the cell surface protein (SlpA) of *C. ammoniagenes* (heterologously fused prepro-transglutaminase gene).

[0078] The amplified fragment of about 1.8 kb was detected by agarose electrophoresis. This fragment was recovered from the agarose gel using EASYTRAP Ver. 2 (Takarashuzo Co. Ltd.) and inserted into SmaI site of pVC7 described in JP-Kokai No. 9-070291 to obtain pVKSPTG1. The nucleotide sequence of the inserted fragment was determined according to the aforementioned method and it was confirmed that the expected fusion gene was constructed.

[0079] The fusion gene of about 1.8 kb for transglutaminase having the pro-structure, which was ligated to the region encoding the N-terminal 25 amino acid residues (signal peptide) of the cell surface protein (SlpA) of *C. ammoniagenes* and which comprised the 5'-upstream region containing the promoter region of the gene of the protein corresponding to PS2 of *C. glutamicum* ATCC13869, was excised by digesting pVKSPTG1 with KpnI and XbaI, and the fragment was recovered using agarose electrophoresis. This fragment was inserted into KpnI-XbaI site of pPK4 described in JP-Kokai No. 9-322774 to construct pPKSTG1. Both plasmids, pVKSPTG1 and pPKSPTG1 contained the promoter derived from PS2 gene of *C. glutamicum* ATCC13869, the signal peptide gene derived from SlpA of *C. ammoniagenes* and the transglutaminase gene derived from *S. mobaraense*.

(3) Conversion to *E. coli* tac promoter

[0080] The primers shown in SEQ ID NO:28 and SEQ ID NO:29 were synthesized based on the sequence of plasmid pKK223-3 (Amersham Pharmacia Co. Ltd.) into which *E. coli* tac promoter had been cloned. The region corresponding to tac promoter was amplified using PCR method from pKK223-3 DNA. The primer shown in SEQ ID NO:29 also comprises the sequence encoding the N-terminal amino acid sequence of the signal sequence of the cell surface protein (SlpA) of *C.*

ammoniagenes in order to construct the fusion gene having the pro-structure part, which contained the signal sequence of the cell surface protein (SlpA) of *C. ammoniagenes* (heterologously fused prepro-transglutaminase gene).

(SEQ ID NO:28) 5'-GGATCCGGAGCTTATCGACTGCACG-3'

(SEQ ID NO:29)

5'-

CGCAGCCAGCGATTTCATGCGTTTCATAATTCTGTTTCCTGTGTGAAATTG

T-3'

SEQ ID NOs:28 and 29 : PCR primers

[0081] The fusion gene for transglutaminase having the additional pro-structure part, which was ligated to the region encoding the N-terminal 25 amino acid residues of the cell surface protein(SlpA) of *C. ammoniagenes* and which contained tac-promoter (heterologously fused prepro-transglutaminase gene), was amplified by performing cross-over PCR with SEQ ID NO:28 and SEQ ID NO:12 using the mixture of 1 μl of PCR solution of the amplified region corresponding to tac-promoter and 1 μl of PCR solution of the amplified region of the gene for transglutaminase having the pro-structure part, which contained the signal sequence of the cell surface protein (SlpA) of *C. ammoniagenes*, as the templates. The amplified fragment of about 1.5 kb was detected by agarose electrophoresis. This fragment was recovered from the agarose gel by EASYTRAP Ver. 2 (Takarashuzo Co. Ltd.) and inserted into SmaI site of the pVC7 as described in JP-Kokai No. 9-070291 to obtain pVTSPTG1. The nucleotide sequence of the inserted fragment was determined according to the aforementioned method and it was confirmed that the expected fusion gene was constructed.

[0082] The fusion gene about 1.5 kb for transglutaminase having the pro-structure part, which was ligated to the region encoding the N-terminal 25 amino acid residues of the cell surface protein(SlpA) of *C. ammoniagenes* and tac promoter, was excised by digesting pVTSPTG1 with KpnI and XbaI and was recovered using agarose electrophoresis. This fragment was inserted into KpnI-XbaI site of pPK4 described

in JP-Kokai No. 9-322774 to construct pPTSPTG1. Both plasmids pVTSPTG1 and pPTSPTG1 contained tac-promoter derived from *E. coli*, the signal peptide gene derived from SlpA of *C. ammoniagenes* and the pro-transglutaminase gene derived from *S. mobaraense*.

(4) Secretion of pro-transglutaminase using the signal sequence of cell surface protein of *C. ammoniagenes*

[0083] *C. glutamicum* ATCC13869 was transformed with the constructed plasmid pVKSPTG1, pVTSPTG1, pPKSPTG1, or pPTSPTG1 and the strains grown on the CM2S agar medium comprising 5 mg/l of chloramphenicol or 25 mg/l of kanamycin were selected. The selected *C. glutamicum* ATCC13869 harboring pVKSPTG1, pVTSPTG1, pPKSPTG1, or pPTSPTG1 was then cultured in the aforementioned MM culture medium comprising 5 mg/l of chloramphenicol or 25 mg/l of kanamycin at 30°C for 48 hours, respectively. After the culture was finished, 10 μl of the supernatant of the culture was subjected to SDS-PAGE and then Western blot was performed using anti-transglutaminase antibody as described in Biosci. Biotechnol. Biochem., 58, 82-87(1994) according to the conventional method. As a result, the similar amount of transglutaminase was confirmed to be secreted for either of the vectors, pVC7 or pPK4. The representative amounts of the secretion are shown in Table 2.

Table 2. The secreted amount of pro-transglutaminase using the signal sequence of cell surface protein of *C. ammoniagenes* ATCC13869

plasmid	protransglutaminase (mg/l)
pPKSPTG1	102
pPTSPTG1	74

[0084] Example 5: Secretory production of pro-transglutaminase using the fusion gene containing the sequence encoding the signal sequence of the cell surface protein of *C. ammoniagenes* and the protransglutaminase derived from *Streptoverticillium*

cinnamoneum IFO12852

(1) Construction of the fusion gene comprising the sequence encoding the signal sequence of the cell surface protein of *C. ammoniagenes* and the sequence encoding the pro-transglutaminase derived from *S. cinnamoneum* IFO12852

[0085] The sequence of the transglutaminase gene of *S. cinnamoneum* IFO12852 has been determined [Japanese Patent Application No.11-295649]. The region from position 1 to position 32 in the amino acid sequence is presumed to be the sequence for the pre-part, from position 33 to position 86 is presumed to be the sequence for the pro-part and from position 87 to position 416 is presumed to be the sequence for the mature transglutaminase sequence. The nucleotide sequence and the entire amino acid sequence encoded by the nucleotide sequence are shown in SEQ ID NO:30 and SEQ ID NO:31. Additionally *Escherichia coli* AJ13669 which had been transformed with the plasmid pUJ-MTG containing the gene has been originally deposited in the National Institute of Bioscience and Human-Technology Agency of Industrial Science and Technology (Now, I Independent Administrative Agency, National Institute of Advance Industrial Science and Technology, Tsukuba Central 6, 1-1, Higashi 1-Chome Tsukuba-shi, Ibaraki-ken, 305-8566 Japan) on Oct. 14, 1999 as FERM P-17602 and has been transferred to the deposit under the Budapest Treaty on August 28, 2000, and the deposit number of FERM BP-7287 has been allotted.

[0086] The region of 3.5 kb covering the full-length of the prepro-transglutaminase gene was firstly excised from pUJ-MTG with restriction enzyme BamHI, and pUCSCTG was generated wherein the region was inserted into BamHI site of pUC19.

[0087] Primers shown in SEQ ID NO:32 and SEQ ID NO:33 were synthesized, and the region of the gene comprising the pro-transglutaminase derived from *S. cinnamoneum* IFO12852 was amplified by PCR method using pUCSCTG as the template as previously described.

(SEQ ID NO:32) 5'-GGC GAT GGG GAA GAG AAG GGG-3'

(SEQ ID NO:33) 5'-GGC GGA TCC TVG CGT CGA GAG GCG TGG ACT GA-3'

SEQ ID NOs:32 and 33: PCR primers

[0088] The region, which contained the 5'-upstream region containing the promoter region of PS2 gene which is the cell surface protein of *C. glutamicum* and the region containing the signal sequence of the cell surface protein SlpA of C. ammoniagenes, was then amplified by performing PCR using the combination of SEQ ID NO:34 and SEQ ID NO:35 from pPKSPTG1 which was constructed in Example 4(2) as the template.

[0089] The primer shown in SEQ ID NO:35 also contained the sequence encoding the N-terminal amino acid sequence of pro-transglutaminase derived from *Streptoverticillium cinnamoneum* IFO12852 in order to construct the fusion gene with the transglutaminase derived from *Streptoverticillium cinnamoneum* IFO12852.

(SEQ ID NO: 34) 5'-TAC GAA TTC GAG CTC GGT ACC-3'

(SEQ ID NO: 35) 5'-CCC CTT CTC TTC CCC ATC GCC TGC CGT TGC CAC AGG TGC GGC C -3'

SEQ ID NO: 34 and 35: PCR primers

[0090] The fragment of the heterologously fused prepro-transglutaminase gene, which was ligated to the signal sequence of the cell surface protein SlpA of *C. ammoniagenes* and the 5'-upstream region comprising the promoter region of PS2 gene, was amplified by performing cross-over PCR with SEQ ID NO: 34 and SEQ ID NO: 33 using the mixture comprising 1 μl of PCR solution of the amplified region encoding the gene for the pro-transglutaminase derived from *C. cinnamoneum* IFO12852 and 1 μl of PCR solution of the amplified region comprising 5'-upstream region containing the promoter region of the PS2 gene and the region containing the signal sequence of the cell surface protein SlpA of *C. ammoniagenes*, as the templates.

[0091] The amplified fragment of about 1.8 kb was detected by agarose electrophoresis. This fragment was digested with EcoRI and BamHI, and then recovered from the agarose gel and inserted into EcoRI-BamHI site of the pUC19 to obtain pUKSPTG2'. The sequence of the inserted fragment was determined according to the aforementioned method and it was confirmed that the fusion gene

was constructed as expected. This pUKSPTG2' was digested with EcoRI and blunt-ended with Blunting Kit (Takarashuzo Co. Ltd.), and XbaI linker (Takarashuzo Co. Ltd.) having the sequence 5'-CTCTAGAG-3' wherein 5'-terminal was phosphorylated was then inserted and re-cyclized to construct pUKSPTG2. The fused preprotransglutaminase gene of about 1.8 kb (the protransglutaminase gene was derived from *S. cinnamoneum* IFO12852) was excised by digesting pUKSPTG2 with XbaI and was recovered using agarose electrophoresis. These fragments were inserted into XbaI site of pPK4 described previously to construct pPKSPTG2.

[0092] The preprotransglutaminase gene having a chimeric pro-structure part, wherein the N-terminal of the pro-structure part was partially replaced by the pro-structure part of *S.mobaraense*, was constructed (the mature transglutaminase gene and the part of the pro-structure part were derived from *S. cinnamoneum* IFO12852).

[0093] First, the fragment of about 1.8 kb containing the prepro-transglutaminase gene of EcoRI-BamHI was excised from the plasmid pPKSPTG1 (for the expression of the pro-transglutaminase derived from S. mobaraense IFO13819) which was constructed in Example 4(2), and the fragment was inserted into EcoRI-BamHI site of pUC19 (pUKSPTG1). The fragment of about 1.2 kb was excised by digesting pUKSPTG1 with AatII, and pUKSPTG2' was also digested with AatII to prepare the fragment of about 3.3 kb removing the fragment of about 1.2 kb. This fragment of about 3.3 kb was ligated to the AatII fragment of about 1.2 kb derived from pUKSPTG1, and clones wherein the AatII fragment was inserted were selected according to the conventional genetic engineering techniques. In order to determine the orientation the inserted AatII fragment in the clones, the clones were serially sequenced and the clones where the fragment was inserted in the desired orientation (for encoding preprotransglutaminase) were selected (pUKSPTG3').

[0094] Moreover the EcoRI site of pUKSPTG3' was also blunt-ended as described for pUKSPTG2' and XbaI linker was inserted to construct pUKSPTG3. Further the 1.8kb XbaI fragment excised from pUKSPTG3 was inserted into XbaI site of pPK4 to construct pPKSPTG3.

(2) Secretion of the protransglutaminase derived from *Streptoverticillium cinnamoneum* IFO12852 using the signal sequence of the cell surface protein from *C. ammoniagenes*

[0095] *C. glutamicum* ATCC13869 was transformed with the plasmid pPKSPTG2 or pPKSPTG3, and the strains which grew on the CM2S agar medium described above comprising 25 mg/l of kanamycin were selected. The selected *C. glutamicum* ATCC13869 harboring pPKSPTG2 or pPKSPTG3 was then cultured respectively in MMTG liquid culture medium (60 g of glucose, 0.4 g of magnesium sulfate heptahydrate, 30 g of ammonium sulfate, 1 g of potassium dihydrogenphosphate, 0.01 g of ferrous sulfate heptahydrate, 0.01 g of manganese(II) sulfate pentahydrate, 450 μg of thiamine hydrochloride, 450 μg of biotin, 0.15 g of DL-methionine, 50 g of calcium carbonate per liter of distilled water, adjusted to pH 7.5) containing 25 mg/l of kanamycin at 30°C for 3 days. After the culture was completed, 10 μl of the supernatant of the culture was subjected to SDS-PAGE and then Western blot analysis was performed according to the conventional method with anti-transglutaminase antibody as previously described. This antibody was an antibody for the transglutaminase derived from *S. mobaraense*, but it also showed the reactivity to the transglutaminase derived from *S. cinnamoneum*. Consequently the secretion of the transglutaminase having the pro-structure part derived from *S. cinnamonieum* IFO12852 was confirmed (about 30 to 50 mg/l).

[0096] Example 6: Cloning of the serine protease (SAMP45) gene, and the construction and evaluation of expression plasmids

(1) Construction of the serine protease (SAMP45) gene having the pro-structure part and the signal sequence of the cell surface protein of *C. ammoniagenes* (heterologously fused prepro-serine protease (SAMP45) gene)

[0097] The sequence of the gene of SAMP45 which is a serine protease produced by *S. albogriseolus* [J. Bacteriol., 179, 430-438(1997)] has already been determined.

The primers shown in SEQ ID NO:36 and SEQ ID NO:37 were synthesized on referring to this sequence and the gene region containing the N-terminal pro-structure part of SAMP45, mature SAMP45 and the C-terminal pro-structure part was amplified using PCR method according to the method described previously.

(SEQ ID NO: 36) 5'-AACGGGGAGAACAGCACGGCCGCCGG-3'

(SEQ ID NO:37) 5'-GGCGAATTCTCCGGCGGGCCGTCACCGGT-3'

SEQ ID NOs:36 and 37: PCR primer

[0098] The region comprising 5'-upstream region containing the promoter region of the gene of the cell surface protein PS2 from *C. glutamicum* and the signal sequence of the cell surface protein SlpA from *C. ammoniagenes* was similarly amplified using PCR method with the combination of SEQ ID NO:38 and SEQ ID NO:39 using pPKSPTG1 constructed in Example 4(2) as the template.

[0099] The primer shown in SEQ ID NO:39 comprises the sequence encoding the N-terminal amino acids of pro-serine protease in order to construct the fusion gene containing the serine protease having the pro-structure part.

(SEQ ID NO:38) 5'-GGCAAGCTTAAATTCCTGTGAATTAGCTGA-3'

(SEQ ID NO:39)

5'-CGGCCGTGCTGTTCTCCCCGTTTGCCGTTGCCACAGGTGCGGCC-3'

SEQ ID NO:38 and 39 : PCR primers to construct the fused pro-serine protease gene

[0100] Then the gene fragment of the heterologously fused prepro-serine protease gene, which was ligated to the signal sequence of the cell surface protein SlpA of *C. ammoniagenes* and to the 5'-upstream region containing the promoter region of PS2 gene, was amplified by performing cross-over PCR with SEQ ID NO:38 and SEQ ID NO:37 using the mixture, as the templates, which comprises 1 μl of PCR solution of the amplified region comprising the gene for the N-terminal pro-structure of SAMP45, mature SAMP45 and the C-terminal pro-structure, and 1 μl of PCR solution of the amplified region comprising the 5'-upstream region containing the promoter region of the PS2 gene and the signal sequence of the cell surface protein SlpA of *C. ammoniagenes*, respectively.

[0101] The amplified fragment of about 3.9 kb was detected by agarose gel electrophoresis. The PCR product was digested with HindIII and EcoRI, then subjected to agarose gel electrophoresis, and the fragment of about 3.9kb was recovered from agarose gel and inserted into HindIII-EcoRI site of the aforementioned pVC7 to obtain pVSS1, respectively. The sequence of the inserted fragment was determined according to the aforementioned method and it was confirmed that the fusion gene was constructed as expected.

(2) Secretion of the serine protease using the signal sequence of the cell surface protein of *C. ammoniagenes*

[0102] *C. glutamicum* ATCC13869 was transformed with the plasmid pVSS1 and the strains which grew on the CM2S agar medium described above comprising 5 mg/l chloramphenicol were selected. The selected *C. glutamicum* ATCC13869 harboring pVSS1 was then cultured in MMTG culture medium comprising 5 mg/l chloramphenicol at 30°C for 70 hours. 1ml of the culture medium was separate into the supernatant of the culture medium and the cells by centrifugation. The cells were suspended in 0.1 M sodium phosphate buffer (pH 7.0). The activity of the serine protease was determined as follows: 50μl of the supernatant of the culture medium or the cell suspension was added to 20 mM sodium phosphate buffer (pH 7.0) containing 0.25 mM Bz-Phe-Val-Arg-pNA (Bachem Co. Ltd.) to give a total amount of 0.6 ml, which was maintained at 30°C for 20 minutes. Thereafter the reaction was terminated upon the addition of 0.4 ml of 50% acetic acid. The absorbance was measured at 410 nm and the amount of p-NA (p-nitroanillide) released was measured to determine the activity. One unit of the enzyme was defined as the amount of enzyme which releases 1 μmol of pNA per one minute. As a result, the activity of serine protease was not detected in the supernatant of the culture medium, but was detected in the cell suspension. Calculating from the values of detected activity and the values of the specific activity reported in the literature [J. Bacteriol., 179, 430-438(1997)], as much as about 9 mg/l of serine

protease was confirmed to be expressed and secreted at the surface of the cell.

[0103] Example 7: Cloning of the proline specific peptidase (svPEP) gene, and construction and evaluation of expression plasmids

(1) Purification and analysis of the N-terminal amino acids of the proline specific peptidase (svPEP) produced by *S. mobaraense* IFO13819

[0104] 800 mL of ISP2 liquid culture medium (4 g of yeast extract, 10 g of malt extract, 4 g of glucose filled up to 1L by water, adjusted to pH 7.3) was placed in a 5L Sakaguchi flask and *S. mobaraense* IFO13819 was inoculated from the plate into the flask and cultured by shaking the flask at 30°C for 48 hours at 120 rpm.

[0105] The culture medium was centrifuged to remove the supernatant of the culture and the cells were harvested. After washing the cells with 20 mM Tris-HCl buffer containing 25 mg/l kanamycin, the resulting cells were suspended in 0.1 M sodium phosphate buffer (pH 7.0) containing 25 mg/l kanamycin. The suspension was shaken on ice for 4 hours and centrifuged to give the supernatant, which was collected. After the supernatant was filter-sterilized using nitrocellulose filter (0.22 μm pore sized, Sartrius Co. Ltd.), the supernatant was passed through the Butyl-Sepharose 4FF (Amersham Pharmacia Co. Ltd.) column (1.6φ×10 cm), which had been pre-equilibrated with 1.5 M ammonium sulfate/ 50 mM phosphate buffer (pH 7.0), using FPLC (Amersham Pharmacia Co. Ltd.) and eluted by the linear gradient of ammonium sulfate 1.5 to 0 M in the same buffer. Fractions containing active components were pooled and passed through Phenyl-Sepharose HP column (1mL, Amersham Pharmacia Co. Ltd.) under the same condition, and the active fractions were pooled and dialyzed overnight against 50 mM sodium phosphate buffer (pH 7.0) at 4°C to give partially purified enzyme solution. The partially purified enzyme solution was subjected to a reversed phase chromatography for further purification. The condition of the reversed phase chromatography was as follows:

HPLC device: pump: HITACHI L-6300, detector: L-4000H

Column : PROTEIN C4 214TP5410(VYDAC Co. Ltd.)

Elution : Elution was effected by a lineal gradient of acetonitrile 24-40 % / 0.1% trifluoroacetic acid (20 min) at room temperature

Flow rate : 1.0 ml/min.

Detection wavelength: 280 nm

[0106] The enzyme sample which was purified under the condition describe above was transferred onto Polyvinylidene-difluoride (PVDF) membrane using Membrane Cartridge (Perkin Elmer Co. Ltd.) and the N-terminal amino acid sequence was analyzed using gas-phase Protein Sequencer PPSQ-10 (Shimazu Seisakusho Co., Ltd.).

[0107] As a result, the N-terminal 20 amino acid residues were determined, which are shown in SEQ ID NO:40.

(SEQ ID NO:40) Gln Ala Asp Ile Lys Asp Arg Ile Leu Lys Ile Pro

1 5 10

Gly Met Lys Phe Val Glu Glu Lys

15 20

(2) Acquisition of the proline-specific peptidase(svPEP) gene derived from *S. mobaraense* IFO13819

[0108] The region which is deduced from the determined N-terminal amino acid sequence of svPEP and which has less degeneracy, Lys-Ile-Pro-Gly-Met-Lys-Phe-Val-Glu-Glu-Lys (SEQ ID NO:41), was selected and the synthetic oligonucleotide shown in SEQ ID NO: 42 was generated. The chromosomal DNA of *S. mobaraense* IFO13819 prepared according to the conventional method was digested with various restriction enzymes which recognize 6-nucleotides sequence and then analyzed by Southern blot hybridization method using this synthetic oligonucleotide as the probe and thereby a single band of about 6 kb was detected by SacI cleavage. Accordingly, the chromosomal DNA of *S. mobaraense* IFO13819 prepared according to the aforementioned method was digested with Sac I and the fragment of about 6 kb was recovered by agarose gel electrophoresis using EASYTRAP Ver. 2 (Takarashuzo

Co. Ltd.). The recovered fragment was inserted in Sac I site of pUC18, which was introduced into the competent cell of Escherichia coli JM109 (Takarashuzo Co. Ltd.), thereby producing a library. The generated library in this way was screened for the strain which harbored the plasmid where the fragment of svPEP gene was cloned, by screening the library through colony hybridization using ^{32}P-labeled synthetic oligonucleotide shown in SEQ ID NO:38 as a probe to obtain the intended gene. The plasmid recovered from this strain was designated as pUMP1.

(SEQ ID NO:42) 5'-AAGATCCCCGGGATGAAGTTCGTCGAGGAGAAG-3'

SEQ ID NO:42 : a probe for svPEP

[0109] The nucleotide sequence of the fragment which was cloned as pUMP1 was determined. The amino acid sequence encoded by this gene was deduced and the previously determined N-terminal amino acid sequence (20 residues) based on the enzyme protein was found, and the entire primary amino acid sequence containing the putative signal sequence and the pro-structure part of svPEP was determined, which is shown in SEQ ID NO:9. In the amino acid sequence, position 1 to 25 is supposed to be the signal sequence, position 26 to 33 is supposed to be the pro-structure and position 34 to 477 is the mature svPEP.

[0110] *Escherichia coli* AJ13669 which was transformed with pUMP1 has been deposited in the National Institute of Bioscience and Human-Technology Agency of Industrial Science and Technology (Now, Independent Administrative Agency, National Institute of Advance Industrial Science and Technology, Tsukuba Central 6, 1-1, Higashi 1-Chome Tsukuba-shi, Ibaraki-ken, 305-8566 Japan) on May 15, 2000 as FERM BP-7160 under the Budapest Treaty.

(3) Construction of the proline specific peptidase (svPEP) gene having the pro-structure part with the signal sequence of the cell surface protein of *C. ammoniagenes* (heterologously fused prepro-proline specific peptidase (svPEP) gene)

[0111] Primers shown in SEQ ID NO:43 and SEQ ID NO:44 were synthesized on referring to the sequence of svPEP determined in Example 7(2), and the gene region

containing the pro-part of svPEP and mature svPEP were amplified by PCR method in the same manner as described previously using pUMP1 constructed in Example 7(2) as the template.

(SEQ ID NO: 43) 5'-GAGGCGGCGTCGATCACCGCCCC-3'

(SEQ ID NO: 44) 5'-GCCAAGCTTGAAGCACCGGGCGGCGGCACCCGG-3'

SEQ ID NO: 43 and 44: PCR primers

[0112] Then the region, which comprises the 5'-upstream region containing the promoter region of PS2 gene which is the gene of the cell surface protein of *C. glutamicum* and the region containing the signal sequence of the cell surface protein SlpA of *C. ammoniagenes*, was amplified by PCR method from pPKSPTG1 constructed in Example 4(2) as the template using the combination of SEQ ID NO:38 and SEQ ID NO:45.

[0113] The primer shown in SEQ ID NO:45 comprises the sequence encoding the N-terminal amino acids of svPEP in order to construct the fusion gene fused to the svPEP having the pro-structure part.

(SEQ ID NO: 38) 5'-GGCAAGCTTAAATTCCTGTGAATTAGGCTGA-3'

(SEQ ID NO: 45)

5'-GGGGCGGTGATCGACGCCGCCTCTGCCGTTGCCACAGGTGCGGCCA-3'

SEQ ID NOs:38 and 45 : PCR primer

[0114] The fragment of the heterologously fused gene of prepro-svPEP, which was ligated to the signal sequence of the cell surface protein SlpA of *C. ammoniagenes* and the 5'-upstream region containing the promoter region of PS2 gene, was then amplified by performing cross-over PCR with SEQ ID NO:38 and SEQ ID NO:44 using the mixture, as the templates, which comprises 1 μl of each PCR solution of the region containing the gene encoding the pro-structure part of svPEP and the mature svPEP, which were amplified respectively, and 1 μl of PCR solution of the amplified region comprising 5'-upstream region containing the promoter region of the PS2 gene and the signal sequence of the cell surface protein SlpA of *C. ammoniagenes*.

(SEQ ID NO:38) 5'-GGCAAGCTTAAATTCCTGTGAATTAGCTTA-3'

(SEQ ID NO:44) 5'-GCCAAGCTTGAAGCACCGGCGGCGGCACCCGG-3'

SEQ ID NO:38 and SEQ ID NO 44 : PCR primer

[0115] The amplified fragment of about 2.1 kb was detected by agarose gel electrophoresis. The PCR fragment was digested with HindIII, and then subjected to agarose gel electrophoresis and the fragment of about 2.1 kb recovered from the agarose gel and inserted into the HindIII site of the pVSS1 described in Example 6(1) to obtain pVSSSP1, respectively. The sequence of the inserted fragment was determined according to the conventional method and it was confirmed that the expected fusion gene was constructed.

(4) Secretion of the proline specific peptidase using the signal sequence of the cell surface protein of *C. ammoniagenes*

[0116] *C. glutamicum* ATCC13869 was transformed with the constructed plasmid pVSSSP1 and the strains which grew on the CM2S agar medium described above comprising 5 mg/l chloramphenicol were selected. The selected *C. glutamicum* ATCC13869 harboring pVSSSP1 was then cultured in MMTG culture medium, described above, comprising 5mg/l chloramphenicol at 30°C for 70 hours. 10 ml of the supernatant of the culture was separated by centrifugation into the supernatant of the culture medium and the cells. The cells were suspended in 0.1 M sodium phosphate buffer (pH7.0). The activity of svPEP was determined as follows: 50 µl of the supernatant of the culture medium or the cell suspension was added to 20 mM sodium phosphate buffer (pH 7.0) containing 0.25 mM Ala-Ala-Pro-pNA (Bachem Co. Ltd.) to give a total amount of 0.6 ml and the mixture was maintained at 30°C for 20 minutes. Thereafter the reaction was terminated upon the addition of 0.4 ml of 50% acetic acid. The absorbance was measured at 410 nm and the amount of p-NA (p-nitroanillide) released was calculated to determine the activity. One unit of the enzyme is defined as the amount of enzyme which releases 1 µmol of pNA per 1 minute. As a result, the activity of svPEP was not detected in the supernatant of the culture medium, but was detected in the cell suspension. Calculating from the

values of the detected activity and the values of the specific activity (35.5u/mg) described in Example 7(1), as much as about 50 mg/l of svPEP was confirmed to be expressed and secreted at the surface of the cell.

(5) Cleavage of the pro-structure part of the transglutaminase having the pro-structure by the serine protease and the proline specific protease expressed and secreted by *C. glutamicum* ATCC13869

[0117] *C. glutamicum* ATCC13869 harboring the secretory expression plasmid pPKSPTG1 for transglutaminase having the pro-structure part described in Example 4(2) was transformed with the constructed plasmid pVSSSP1, and the strains grown on the aforementioned CM2S agar medium comprising 5 mg/l of chloramphenicol and 25 mg/l kanamycin were selected. Then the selected *C. glutamicum* ATCC13869 harboring pVSSSP1 and pPKSPTG1 was cultured in MMTG culture medium, described above, comprising 5 mg/l chloramphenicol and 25 mg/l kanamycin at 30°C for 70 hours.

[0118] After the culture was finished, 10 μl of the supernatant of the culture was subjected to SDS-PAGE and then Western blot analysis was performed with anti-transglutaminase antibody previously described according to the conventional method. As a result, it was confirmed that SAMP45 and svPEP were normally expressed and secreted, and that the pro-structure part was cleaved from the transglutaminase having the pro-structure part which had been also secreted, thereby the secretion of the transglutaminase having the similar molecular weight to that of the naturally occurring mature transglutaminase was confirmed.

[0119] The transglutaminase activity was tested for the supernatant by the hydroxamate method previously described, which confirmed that it contained the similar specific activity (about 20 U/mg) to that of the naturally occurring transglutaminase.

[0120] Additionally, it was semi-dry blotted onto polyvinylidene difluoride (PVDF) membrane according to the method previously described after SDS-PAGE.

After blotting, the PVDF membrane was stained with Coomassie Brilliant Blue, de-stained and air-dried. The portion containing the mature transglutaminase was excised and analyzed for the N-terminal amino acid sequence using a protein sequencer. As a result, it was confirmed that it had the same N-terminal sequence as the naturally occurring transglutaminase derived from *S.mobaraense* starting Asp located at position 77, which is shown in SEQ ID NO:6.

[0121] Example 8: Secretory production of human epidermal growth factor (hEGF) using the fusion gene containing the sequence encoding the signal sequence of the cell surface protein from *Corynebacterium ammoniagenes* ATCC6872 and the sequence encoding human epidermal growth factor

(1) Construction of hEGF gene containing the signal sequence of the cell surface protein of *Corynebacterium glutamicum* ATCC13869

[0122] The sequence of the gene of the cell surface protein of Corynebacterium glutamicum, PS2, has been already determined [Mol. Microbiol., 9, 97-109 (1993)]. The primers shown in SEQ ID NO:46 and NO:47 were synthesized on referring to this sequence. The gene region comprising the 5'-upstream region and the region encoding N-terminal 44 amino acids residue of the protein correspond to PS2 was amplified using PCR method from the chromosomal DNA of *Corynebacterium glutamicum* ATCC13869 which had been prepared according to the method of Saito & Miura [Biochem. Biophys. Act., 72, 619 (1963)]. The primer shown as SEQ ID NO:46 contained KpnI site at its 5'-terminal which was required to insert the region into a plasmid.

(SEQ ID NO:46) 5'-CTCGGTACCCAAATTCCTGTGAATTAGCTGATTTAG-3'

(SEQ ID NO:47) 5'-GTTGAAGCCGTTGTTGATGTTGAA-3'

SEQ ID NOs:46 and 47: PCR primer

[0123] On the other hand, the primers shown in SEQ ID NO:48 and NO:49 were synthesized. The region encoding hEGF was amplified by PCR method from plasmid pT13SΔhIL2-KS-hEGF(H3) (JP Kokai No. 64-2583) which contains the gene

sequence for hEGF. *Escherichia coli* AJ12354 transformed with plasmid pT13SΔhIL2-KS-hEGF(H3) containing the gene was originally deposited at Independent Administrative Agency, National Institute of Advance Industrial Science and Technology (Tsukuba Central 6, 1-1, Higashi 1-Chome Tsukuba-shi, Ibaraki-ken, 305-8566 Japan) on November 20, 1987 and has been transferred to the international deposit under the Budapest Treaty on March 18, 2002, and the deposit number of FERM BP-7966 has been allotted.

[0124] The primer shown as SEQ ID NO:48 contains the sequence encoding the C-terminal amino acids of the signal sequence of PS2 in order to construct the fusion gene with the region which comprises 5'-upstream region and which also comprises the region encoding 44 N-terminal amino acid residues of the protein corresponding to PS2.

(SEQ ID NO:48) 5' - AACATCAACAACGGCTTCAACAATTCCGATTCTGAGTGCCCT-3'

(SEQ ID NO:49) 5'-CGGCCACGATGCGTCCGGCG-3'

SEQ ID NOs: 48 and 49 : PCR primer

[0125] Then, the fusion gene, where hEGF was ligated to the region comprising 5'-upstream region and the region encoding N-terminal 44 amino acid residues of the cell surface protein of *Corynebacterium glutamicum*, was amplified by performing cross-over PCR with SEQ ID NO:46 and SEQ ID NO:49 using the mixture, as the template, containing 1 μl of PCR reaction solution with the region comprising 5'-upstream region and the region encoding N-terminal 44 amino acid residues of the cell surface protein of *Corynebacterium glutamicum* and 1 μl of PCR reaction solution containing amplified hEGF gene region. The fragment of about 0.9kb was detected by agarose gel electrophoresis. The fragment was recovered from agarose gel by using EASY TRAP Ver. 2 (Takar4ashuzo Co. Ltd.). The recovered DNA was cleaved by KpnI and BamHI (Takar4ashuzo Co. Ltd.), purified by DNA Clean-UP system (Promega), and inserted to KpnI-BamHI sited of plasmid pPK4 described in

JP Kokai No. 9-322774 to obtain pPKEGF. The nucleotide sequence of the inserted fragment was determined by using Dye Terminator Cycle Sequencing kit (PE Applied Biosystems) and DNA Sequencer 373A (PE Applied Biosystems) to confirm that the expected fusion gene had been constructed.

(2) Construction of hEGF gene containing the signal sequence of *Corynebacterium ammoniagenes* ATCC6872

[0126] The primers shown in SEQ ID NO:24 and SEQ ID NO:50 were synthesized on referring to the sequence of the gene of the cell surface protein (SlpA) [JP-Kokai No. 10-108675] of *C. ammoniagenes* and the region comprising the 5'-upstream region containing the promoter region of the cell surface protein (SlpA) gene and the region encoding its N-terminal 25 amino acid residues was amplified using PCR method from the chromosomal DNA of *C. ammoniagenes* prepared according to the method described in Example 8(1). The primer shown in SEQ ID NO:50 also contained the sequence encoding the N-terminal amino acids of hEGF in order to construct the fusion gene fused with hEGF.

(SEQ ID NO: 24) 5'-GCCCAGAAGCCCAAAATTGAGATTT-3'

(SEQ ID NO: 50) 5'-
AGGGCACTCAGAATCGGAATTTGCCGTTGCCACAGGTGCGGCC-3'

SEQ ID NOs: 24 and 50: PCR primer

[0127] The primers indicated in SEQ ID NO:51 and SEQ ID NO:49 were synthesized and the region encoding hEGF was amplified from plasmid pT13SDhIL2-KS-hEGF(H3) (JP Kokai No. 64-2583) containing hEGF gene sequence.

(SEQ ID NO:49) 5'-CGGCCACGATGCGTCCGGCG-3'

(SEQ ID NO:51) 5'-AATTCCGATTCTGAGTGCCCT-3'

SEQ ID NOs: 49 and 51: PCR primer

[0128] The fusion gene, where hEGF gene was ligated to the 5'-upstream region and the region encoding the N-terminal 25 amino acid residues of the cell surface

protein (SlpA) of *C. ammoniagenes*, was amplified by performing cross-over PCR with SEQ ID NO:49 and SEQ ID NO:24 using the mixture, as the templates, containing 1 μl of PCR solution of the amplified 5'-upstream region and the region encoding the N-terminal 25 amino acid residues of the cell surface protein (SlpA) of *C. ammoniagenes* and 1 μl of PCR solution for hEGF gene region.

[0129] Additionally, the primers indicated in SEQ ID NO:52 and SEQ ID NO:53 were synthesized, and 5'-upstream region of the protein corresponding to PS2 was amplified by PCR method using the plasmid pKEGF obtained in Example 8 (1) as the template. The primer shown in SEQ ID NO:53 contained the 3'-terminal sequence of 5'-upstream region for the protein corresponding to PS2 and the region encoding the N-terminal amino acids of the signal sequence of the cell surface protein (SlpA) from *Corynebacterium ammoniagenes*.

(SEQ ID NO:52) 5'-GAATTCGAGCTCGGTACCCA-3'

(SEQ ID NO:53)

5'-AGCGATTTCATGCGTTTCATAGAGGCGAAGGCTCCTTGAA-3'

SEQ ID NOs: 52 and 53 : PCR primer

[0130] On the other hand, the gene hEGF gene region containing the signal sequence of the cell surface protein (SlpA) from *Corynebacterium ammoniagenes* was amplified by using the primers indicated in SEQ ID NO:54 and SEQ ID NO:49 prepared based on the hEGF fusion gene comprising the signal sequence and the 5'-upstream region of the cell surface protein (SlpA) from *Corynebacterium ammoniagenes*. For PCR, Pyrobest DNA polymerase (Takarashuzo Co. Ltd.) was used and the reaction condition followed the instructed protocol.

(SEQ ID NO:49) 5'-CGGCCACGATGCGTCCGGCG-3'

(SEQ ID NO:54) 5'-ATGAAACGCATGAAATCGCTGGC-3'

SEQ ID NOs: 49 and 54 : PCR primer

[0131] The fusion gene of hEGF, which was ligated to the region encoding the N-terminal 25 amino acid residues of the cell surface protein (SlpA) of *Corynebacterium*

ammoniagenes and to the 5'-upstream region encoding the protein corresponding to PS2 of *Corynebacterium glutamicum*, was then amplified by performing cross-over PCR with SEQ ID NO:52 and SEQ ID NO:49 using the mixture comprising 1 μl of PCR solution of the amplified 5'-upstream region for the protein corresponding to PS2 of *Corynebacterium glutamicum* and 1 μl of PCR solution of the amplified region of the gene for hEGF having the signal sequence of the cell surface protein (SlpA) of *Corynebacterium ammoniagenes*. For PCR, Pyrobest DNA polymerase (Takarashuzo Co. Ltd.) was used and the reaction condition followed the instructed protocol. The amplified fragment of about 0.9kb was detected by agarose gel electrophoresis. This fragment was recovered from the agarose gel using EASYTRAP Ver. 2 (Takarashuzo Co. Ltd.), digested with KpnI and BamHI (Takarashuzo Co. Ltd.) and was purified with DNA Clean-UP system (Promega). The fragment was inserted into KpnI-BamHI site of the plasmid pPK4 described in JP Kokai No. 9-322774 to obtain pPSEGF. The nucleotide sequence of the inserted fragment was determined by using Dye Terminator Cycle Sequencing kit (PE Applied Biosystems) and DNA Sequencer 373A (PE Applied Biosystems) to confirm that the expected fusion gene had been constructed.

(3) Generation of hEGF producing strains

[0132] *Corynebacterium glutamicum* ATCC13869 was transformed with the hEGF expression plasmid pPSEGF constructed in (2) by electroporation method to obtain kanamycin resistant strains. The obtained strains was cultured with shaking at 30°C for 3 days with MMTG liquid medium (60g of glucose, 0.4g of magnesium sulfate heptahydrate, 30g of ammonium sulfate, 1g of potassium dihydrogenphosphate, 0.01 g of ferrous sulfate heptahydrate, 0.01g of manganese(II) sulfate pentahydrate, 450μg of thiamine hydrochloride, 450μg of biotin, 0.15g of DL-methionine, 50g of calcium carbonate per liter of distilled water, adjusted to pH 7.5) containing 25mg/ml of

kanamycin. The cells were removed by centrifugation and 10μl of the supernatant of the culture was subjected to SDS-PAGE. The commercially available hEGF (PEPRO TECHEC LTD) was simultaneously subjected to electrophoresis as a standard, and Coomassie Brilliant Blue (CBB) staining was carried out. The results showed that the band was detected at the position corresponding to the same mobility as the standard. There were few other impure proteins in the culture supernatant.

(4) Determination of the amount of hEGF produced by the hEGF producing strain harboring the plasmid pPSEGF

[0133] The culture supernatant of the hEGF producing strain was analyzed with HPLC column (YMC-AP203Ca300A.C18, particle size 5μm, diameter 4.6mm x length 250mm), using 0.1% TFA/24% acetonitrile, 0.1%TFA/44 acetonitrile as buffers, 1%/min. linear gradient, flow rate 1.0 ml/min and detection at 280nm. The quantification was carried out by comparing the peak area with the area observed when the hEGF standard was analyzed, which revealed the value of about 100mg/L.

(5) Determination of the biological activity of hEGF secreted by hEGF producing strain harboring the plasmid pPSEGF

[0134] The EGF activity in the culture supernatant of the hEGF producing strain was determined. MCF-7 cells (A.V. Krishman, Journal of Bone and research, 6, 1099-1107, 1991) were placed on 96-well plate at a initial cell density of 1×10^4 per well and the 2-fold serial dilution of the culture supernatant of the EGF producing strain was added to the wells. The uptake of thymidine was determined after 72 hours. The activity was 1.4×10^9 U/ml as calculated by comparing with the value of hEGF standard, 10^7U/mg.

(6) Analysis of N-terminal amino acid sequence of hEGF secreted by the hEGF

producing strain harboring the plasmid pPSEGF

[0135] 120μl of the culture supernatant of hEGF producing strain was applied to HPLC column, and isolated by using the condition described for quantification by HPLC. The peak corresponding with the elution position of the standard hEGF was collected. The determination was carried out by using gas phase amino acid sequencer PPSQ-10 (Shimadzu Co.). The results are as follows: Asn for the first residue; Ser for the second residue; Asp for the third residue; Ser for the fourth residue, respectively from the N-terminal. The results corresponded to the N-terminal amino acid sequence of hEGF indicated in SEQ ID NO:55.

(7) Determination of the molecular weight of hEGF secreted by the hEGF producing strain harboring the plasmid pPSEGF

[0136] 120μl of the culture supernatant of hEGF producing strain was applied to HPLC column, and isolated by using the condition described in (4).

[0137] The eluted hEGF peak was collected. The sample was applied again to the same HPLC for fractionation, which confirmed that the sample exhibited one peak in HPLC. The sample was subjected to mass spectroscopy. The determination was carried out by using MALDI-TOFMS (Matrix assisted laser deionization - time of flight mass spectrometer) MALDI IV (Shimadzu Co.). The average of two measurements was 6176. The result was within the limit of error of theoretical value 6217 which had been calculated for the molecular weight of hEGF, assuming that there were three S-S bonds in the molecule. Thus, hEGF produced by the EGF producing strain prepared by the described method was confirmed to have the expected amino acid sequence and structure.

Example 9: Construction of *Corynebacterium glutamicum* AJ12036 improved in the secretion of heterologous proteins, construction of cell surface protein (PS2) gene

disruption strain derived from AJ12036, and the estimation of the production of heterologous protein using these mutant strains

(1) Production of cell surface protein (PS2) gene disruption strain from *Corynebacterium glutamicum* AJ12036

[0138] Streptomycin (Sm) resistant strain AJ12036 had been bred from *Corynebacterium glutamicum* ATCC13869 and AJ12036 has been used as a host for gene recombination with *Corynebacterium glutamicum* (U.S. Patent No. 4,822,738). *Corynebacterium glutamicum* (formerly, *Brevibacterium lactofermentum*) AJ12036 was deposited on the National Institute of Microbial Technology, Agency of Industrial Science and Technology (Now, Independent Administrative Agency, National Institute of Advance Industrial Science and Technology, Tsukuba Central 6, 1-1, Higashi 1-Chome Tsukuba-shi, Ibaraki-ken, 305-8566 Japan) on March 26, 1984 as FERM BP-734.

[0139] Since it was revealed that AJ12036 strain slightly secreted the cell surface protein (PS2) into the culture medium, it was supposed that the secretion efficiency of proteins could be further improved by conducting the gene disruption such that it would become completely defective in producing PS2. Therefore, the complete PS2 gene defective strain was constructed using homologous recombination as described below.

[0140] The primers described below were synthesized on referring the chromosomal DNA of *Corynebacterium glutamicum* ATCC13869 prepared according to the method of Saito and Miura [Biochim. Biophys. Acta., 72, 619 (1963)] and PCR was carried out with the combination of SEQ ID NOs:56 and 57, and SEQ ID NOs:58 and 59. The sequence of PS gene of *Corynebacterium glutamicum* ATCC13869 was described in U.S. Patent No. 5,547,864, and a part of the coding region and its 5'-upstream gene sequence were described in SEQ ID NO:4.

[0141] Crossover-PCR with the amplified each fragment and the combination of the primers of SEQ ID NO:56 and NO:59 was conducted to amplify ΔPS2 fragment

where the promoter region and the N-terminal region of the coding region of PS2 gene were deleted from PS2 gene. This fragment was cloned into SmaI site of pUC19 to construct pUΔPS2. pUΔPS2 was digested with KpnI and XbaI to excise ΔPS2 fragment and, pHSΔPS2 was constructed by inserting the fragment into KpnI-XbaI site of pHS4 (U.S. Patent No. 5,616,489) which is a temperature sensitive plasmid vector derived from pHM1519. *Escherichia coli* AJ12570 transformed with the plasmid pHS4 was deposited in National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology, Ministry of International Trade and Industry (Now, Independent Administrative Agency, National Institute of Advance Industrial Science and Technology, Tsukuba Central 6, 1-1, Higashi 1-Chome Tsukuba-shi, Ibaraki-ken, 305-8566 Japan) on October 11, 1990 as FERM BP-3523.

(SEQ ID NO:56) 5'-act ggg agg cta tct cca tt-3'

(SEQ ID NO:57) 5'-atc gat ctg atc acg tta ca-3'

(SEQ ID NO:58) 5'-tgt aac gtg atc aga tcg att cac tgg tcg aca ccg ttg a-3'

(SEQ ID NO:59) 5'-acg gaa gct acc ttc gag gt-3'

SEQ ID NO:56 to SEQ ID NO:59 : PCR primer

[0142] pHSΔPS2 was introduced into AJ12036 by electroporation, and the complete PS2 gene defective strain was obtain by homologous recombination described in Japanese Patent No. 2763054. This strain was designated as YDK010 strain.

(2) Estimation of secretory production of heterologous proteins using *Corynebacterium glutamicum* AJ12036 and cell surface protein (PS2) gene disruption strain derived from AJ12036

[0143] The protransglutaminase expression plasmid pPKSPTG1, which was described in Example 4 (2), was introduced into AJ12036 and YDK010 strains to obtain the transformants. These transformants and the control strain obtained by

introducing pPKSPTG1 into the wild type *Corynebacterium glutamicum* ATCC13869, were used to estimate the amount of secretory production.

[0144] Similarly, the amount of secretory production was estimated for SAMP45 secretory expression plasmid pVSS1, for svPEP secretory expression plasmid pVSSSP1 and for hEGF secretory expression plasmid pPSEGF after obtaining the transformants from AJ12036 strain and YDK010 strain, respectively.

[0145] The strains grown overnight on the CM2S agar medium comprising 25mg/l of kanamycin at 30°C were inoculated into large tubes containing 4ml of MMTG medium (Glucose 60g/L, $MgSO_4 \cdot 7H_2O$ 1g/L, $MnSO_4 \cdot 4H_2O$ 1g/L, $FeSO_4 \cdot 7H_2O$ 1g/L, $(NH_4)_2SO_4$ 30g/L, KH_2PO_4 1.5g/L, VB1 · HCl 450μg/L, Biotin 450μg/L, DL-Met 0.15g/L, pH7.5) supplemented with 5% $CaCO_3$ and 25 μg/ml kanamycin, for 3 days at 30°C.

Table 3. The amount of extracellularly produced protransglutaminase by the mutant strains

Producing Strain	Protransglutaminase
ATCC13869/pPKSPTG1	235 mg/L
AJ12036/pPKSPTG1	680
YDK010/pPKSPTG1	700

Table 4. The amount of extracellularly produced SAMP45 by the mutant strains

Producing Strain	SAMP45
ATCC13869/pVSS1	9 mg/L
AJ12036/pVSS1	20
YDK010/pVSS1	22

Table 5. The amount of extracellylarly produced svPEP by the mutant strains

Producing Strain	svPEP
ATCC13869/pVSSSP1	50 mg/L
AJ12036/pVSSSP1	130
YDK010/pVSSSP1	150

Table 6. The amount of extracellularly produced hEGF by the mutant strains

Producing Strain	hEGF
ATCC13869/pPSEGF	100 mg/L
AJ12036/pPSEGF	280
YDK010/pPSEGF	290

[0146] As can be seen in Table 3 to Table 6, the remarkable improvement in the amount of the production of SAMP45, svPEP and hEGF by changing the host from the wild type to the streptomycin resistant strain AJ12036. However, only a slight improvement in the amount of secretory production was observed by completely deleting the cell surface protein (PS2) gene from AJ12036 strain. The negative effect caused by the competitive inhibition of PS2 secretion on the secretion of protransglutaminase or hEGF was not significantly observed in these cases. However, the secretion of PS2 was not observed at all in the complete PS2 gene defective strain, which contributed to the reduction of undesired contaminated proteins in the culture medium. This is a merit during the purification of protransglutaminase or hEGF.

[0147] Example 10: Effective factors in culturing on the secretion of protransglutaminase

[0148] The transformant obtained by transforming *Corinebacterium glutamicum* YDK010 strain with the previously described pPKSPTG1 was used for estimating the

culture condition for the secretory production of protransglutaminase.

[0149] The strain grown overnight on the CM2S agar medium comprising 25mg/l of kanamycin at 30°C were inoculated into 500ml Sakaguchi flask containing 20ml of CM2S liquid medium, and was cultured overnight at 30°C. This was used for seed culture.

[0150] The effects of adding $CaCl_2$ was estimated in S-type Jar containing MMTG liquid medium (Glucose 60g/L, $MgSO_4 \cdot 7H_2O$ 1g/L, $MnSO_4 \cdot 4H_2O$ 1g/L, $FeSO_4 \cdot 7H_2O$ 1g/L, $(NH_4)_2SO_4$ 30g/L, KH_2PO_4 1.5g/L, VB1 · HCl 450μg/L, Biotin 450μg/L, DL-Met 0.15g/L, pH7.5) as the basal medium supplemented with 25μg/ml kanamycin. 300ml medium was placed in the flask. The amount of seeding was 5% (15ml) and the dissolved oxygen concentration was controlled at 3% or less. The culture was carried out at 30°C for 3 days.

[0151] After the culturing, 10μl of the culture supernatant was subjected to SDS-PAGE and Western blotting was carried out using the above-described anti-transglutaminase antibody according to the conventional method. The results showed the effect of adding calcium in that about 1.3- to 2-fold increase in secretion amount was observed in $CaCl_2$ adding groups compared to the non-adding group.

Table 7. Effect of Calcium ion on secretory production of protransglutaminase

CaCl2 (g/L)	Accumulation of protransglutaminase (mg/L)	Relative ratio
0	460	1
0.25	610	1.3
0.5	790	1.7
1.0	810	1.8
2.0	930	2.0

[0152] The conditions for aeration and stirring were further studied using the MMTG medium containing 0.2g/L $CaCl_2$, which revealed that the better results were obtained by controlling the dissolved oxygen concentration at 3% which is the

measuring limit, or less (Table 8).

Table 8. Effect of the concentration of dissolved oxygen on the secretory production of protransglutaminase

Dissolve oxygen concentration	Accumulation of protransglutaminase (mg/L)	Relative ratio
Below 3%	930	1.43
Controlling at 3%	810	1.25
Controlling at 5%	650	1

[0153] According to the present invention, useful proteins, for example, heterologous proteins such as transglutaminase or human epidermal growth factor, can be produced in a large amount and can be efficiently extracellularly secreted (secreto-production) by coryneform bacteria. The protein produced according to the methods of the present invention are secreted into the culture medium, which make it possible to easily recover the protein from the culture media on a large scale using appropriate known methods.